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Effectiveness of Intermediate-Dose Methotrexate and High-Dose 5-Fluorouracil as Sequential Combination Chemotherapy in Refractory Breast Cancer and as Primary Therapy in Metastatic Adenocarcinoma of the Colon[1]

Glenn Tisman,* and Show Jen Grace Wu[2]

SUMMARY

Many studies reported that pretreatment with methotrexate followed by 5-FU resulted in the greatest tumor cell killing. Our preliminary laboratory studies confirmed the possibility of drug synergism in the L1210 cell line, but not in human bone marrow cells. Thirteen patients with metastatic adenocarcinoma of the breast and seven patients with metastatic adenocarcinoma of the colon were treated with an innovative approach in which methotrexate preceded 5-FU administration in an attempt to cause drug synergism and prevent drug antagonism. All patients with breast cancer and two patients with colon cancer had been extensively pretreated with multiple drugs. Of the cancer patients so treated, three (23%) with breast cancer and two (28%) with colon cancer demonstrated objective tumor response. In addition to these patients, six (46%) with breast cancer and three (43%) with colon cancer demonstrated subjective improvement as manifested by total pain relief and reduction in CEA titer. The preliminary results reported in this study suggest that sequential utilization of intermediate doses of methotrexate, followed by high doses of 5-FU, is an effective combination chemotherapy for patients with breast and colon malignancies.

[Cancer Treat Rep 64:829–835, 1980]

There have been reports (1) that methotrexate and 5-FU, when given together, do not demonstrate additive chemotherapeutic activity, thereby suggesting drug antagonism. Work by Bertino et al (2) demonstrated that rats containing the Sarcoma 180 cell line, when treated with methotrexate 2 hours before 5-FU, lived significantly longer than if they received both drugs together or if they received methotrexate after 5-FU. In addition, independent work by Moran[3] suggests that 5-FU may antagonize the activity of methotrexate by preventing oxidation of reduced folate by thymidylate synthetase mediated 2′deoxyuridine-5′-monophosphate (dUMP) conversion to thymidine-5′-monophosphate. Other biochemical work by Cadman et al (3) suggested that pre-incubation of tumor cells with methotrexate directed more 5-FU incorporation into L1210 tumor cell RNA. In addition, by using in vitro cloning techniques Cadman et al (4) were able to show that a 3-hour pretreatment with methotrexate followed by 5-FU resulted in drug synergism in L1210 cell killing.

Later, Tisman and Wu (5) confirmed these studies and showed the absence of such enhancement of 5-FU uptake by normal human bone marrow cells (fig 1). These data suggested an enhanced therapeutic index when these two drugs were used in sequence. Nayak et al (6) suggested that incorporation of 5-FU into tumor cell RNA might be re-

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[3]Moran R. Personal communication, 1978.

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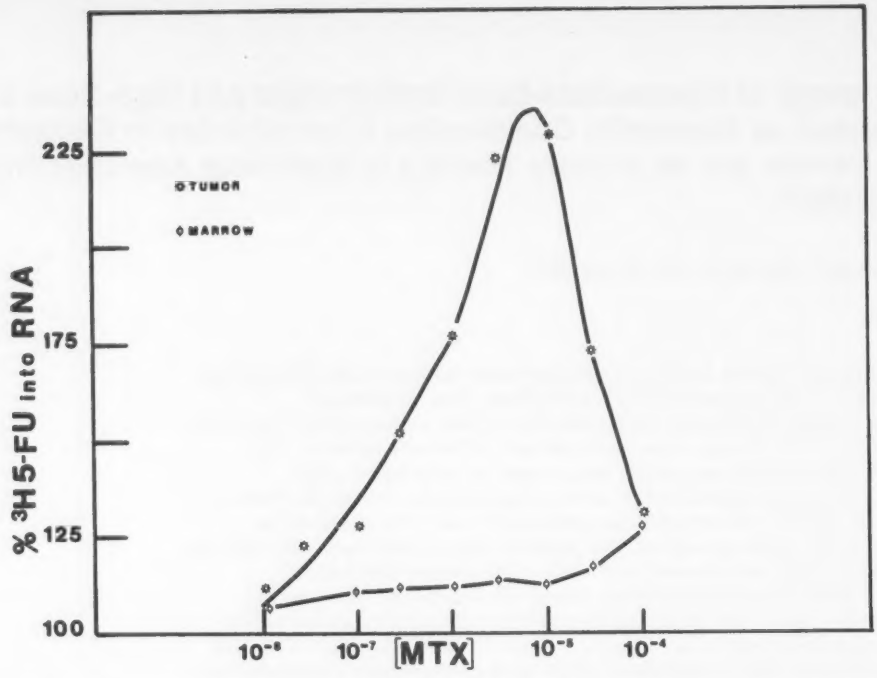

FIGURE 1.—Enhanced incorporation of tritiated 5-FU into L1210 cell RNA, but not normal human bone marrow cell RNA, caused by pre-incubation for 3 hrs with varying concentrations of methotrexate. This suggests that at least for murine tumor cells, pre-incubation with methotrexate may have more of a deleterious effect by forcing more 5-FU into RNA when compared to normal human bone marrow cells, thereby possibly enhancing the therapeutic index of 5-FU. Whether this occurs in mammalian tumor cells is yet to be determined.

sponsible for more cell death than had previously been recognized. Studies by Santi et al (7,8) also suggested that methotrexate could act similar to N^{5-10} methylene tetrahydrofolic acid and thereby form a stable ternary complex between FdUMP, methotrexate, and thymidylate synthetase, thereby possibly enhancing 5-FU inhibition of this enzyme.

All of the biochemical hypotheses and laboratory results seemed to support drug synergism between 5-FU and methotrexate against tumor, but not against normal host bone marrow cells by pretreatment with methotrexate. In an attempt to explore the therapeutic advantage of the drugs, we treated 20 patients with various malignancies with a combination of intermediate-dose methotrexate followed in 1 hour by a high-dose 5-FU followed in 24–30 hours by leucovorin rescue. We also investigated the toxicity of this theoretically synergistic combination of drugs with a theoretically enhanced therapeutic index.

MATERIALS AND METHODS

In Vitro Studies

6-^{3}H5FU (tritium labeled 5-FU), SA = 3 curies/mmol, was purchased from Amersham/Searle, Arlington Heights, IL. Hanks' balanced salt solution was purchased from Grand Island Biological Corp, Grand Island, NY, and methotrexate was purchased from Lederle, Pearl River, NY.

Bone Marrow Cell Culture

Short-term bone marrow culture was carried out essentially as previously described (9,10). Approximately 10 ml of human bone marrow was aspirated into a 30-ml syringe containing 10 ml of tris-buffered (0.06 M, pH 7.4) Hanks' balanced salt solution plus 100 mg of heparin. After aspiration, the bone marrow suspension was washed and the resuspension cells were immediately passed through a 21-gauge

hypodermic needle for a total of four passes. The cells were then centrifuged at room temperature at 500 g × 10 minutes. The supernatant fluid was discarded and a sufficient quantity of tris-buffered Hanks' balanced solution (THBSS)-autologous serum (ratio 3:1) was added to yield a concentration of 1–12 × 10^6 nucleated cells/0.3-ml aliquots of cell suspension as needed for each culture tube.

Bone marrow cells were cultured in 10-ml vacutainer tubes. All experiments were done in triplicate. Each tube contained 0.3 ml of marrow cell suspension. Except for controls, 0.1 ml of different concentrations of methotrexate stock solutions (methotrexate was dissolved in THBSS) were added to appropriate culture tubes.

After methotrexate was added to the culture tubes, the final volume of each tube was adjusted to 0.9 ml with THBSS. Cultures were incubated in triplicate for 4 hours at 37°C in a 7.5% CO_2 in air atmosphere. One hour before incubation was stopped 0.1 ml of ^{3}H5FU (20 microcuries/ml) was added. At the end of the 4-hour incubation, the culture was terminated by adding 2 ml of cold THBSS. The washed cells were then subjected to shock lysis, TCA precipitation, or PCA extraction. The desired portions of either precipitates or extracts were then prepared for scintillation counting as previously described.

L1210 Cell Cultures

All of the media and chemicals used to maintain murine L1210 leukemia cell growth were purchased from Grand Island Biological Corp. Mouse leukemia L1210 cells were adapted to grow and were maintained routinely in RPMI-1640 medium supplemented with 14% heat inactivated (56°C for 1 hour) dialyzed fetal bovine serum, glutamine (0.29 mg/ml), penicillin (100 units/ml), fungizone (0.25 μg/ml), and streptomycin (100 μg/ml).

The cells were transplanted either every 3 or 4 days and only log-phase (approximately 2 days after cell transplantation) L1210 leukemia cells were used for short-term culture experiments. The short-term leukemia cultures were carried out exactly as short-term bone marrow cultures, except that: (*a*) RPMI-1640 was used in place of THBSS; (*b*) autologous human serum was replaced by fetal calf serum; and (*c*) the two shock lysis steps (to remove hemoglobin from human bone marrow cells) were omitted when dealing with L1210 cells.

In Vivo Studies

Patient selection.—Twenty patients were selected for treatment with a combination of intermediate-dose methotrexate and high-dose 5-FU with leucovorin rescue. Of these patients there were 13 with metastatic adenocarcinoma of the breast, all of whom had been refractory to either the Bonadonna (11) or 5-FU, doxorubicin (Adriamycin), and cyclophosphamide (FAC) (12) protocol given either as adjuvant therapy for stage II disease or as primary therapy for metastatic disease. In addition, patients were also refractory to glucocorticoids or appropriate antiestrogen therapy with tamoxifen. Of the 13 patients, five were estrogen receptor positive, six were estrogen receptor negative, and two had not had estrogen receptor determinations done on metastatic tumor tissue. Two patients required an initial reduction of the loading methotrexate dose to 200 mg/m², because of an initial low creatinine clearance. All patients with refractory breast cancer had been pushed to maximum drug tolerance as manifested by leukopenia with either cyclophosphamide, methotrexate, and 5-FU (CMF) or FAC chemotherapy. There were seven patients with metastatic adenocarcinoma of the colon; of these, two had received prior chemotherapy including either 5-FU or methotrexate and one had received prior radiation therapy alone.

Intermediate-Dose Methotrexate and High-Dose 5-Fluorouracil Treatment Schedule

The standard intermediate-dose methotrexate and high-dose 5-FU chemotherapy protocol with leucovorin rescue was given once every 3–4 weeks and consisted of the administration on an outpatient basis of 1500 mg/m² of methotrexate as an iv bolus, followed in 1 hour by the iv administration of 1500 mg/m² of 5-FU. Patients were seen at 18, 24, and 48, and possibly 72, hours after administration of methotrexate for serum methotrexate determinations (13). Leucovorin rescue with 15 mg orally every 6 hours was started between 24 and 30 hours after the initiation of the iv administration of methotrexate. Rescue was continued for between six and eight doses; however, in three instances it was necessary to administer leucovorin for as many as 18 doses. Leucovorin was usually continued for two additional doses after the serum methotrexate level fell to between 1 and 5 × 10^{-8} M at 48 hours (*see* table 1). All chemotherapy was administered on an outpatient basis, except for three courses in which leucovorin rescue could not be given orally because of nausea and vomiting. However, in subsequent treatments of these patients, leucovorin administration did not require hospitalization. The courses were repeated every 3–4 weeks, usually depending

TABLE 1.—Serum methotrexate concentration (mols/liter)

Hrs after methotrexate administration	No. of patients	Range	Average
½	12	7×10^{-4} to 3×10^{-5}	3.5×10^{-4}
1	12	1.9×10^{-4} to 4.6×10^{-6}	8.5×10^{-5}
18	17	3.1×10^{-6} to 8×10^{-8}	1.3×10^{-6}
24	15	8×10^{-7} to 5.5×10^{-8}	5.0×10^{-7}
48	14	4×10^{-7} to 1.3×10^{-8}	1.1×10^{-7}

upon the wbc count returning to > 4500/mm³ and all patients receiving the chemotherapy were required to have a creatinine clearance > 60 ml/minute, except for the two previously mentioned patients with metastatic adenocarcinoma of the breast. In addition, all patients forced fluids by mouth in a home hydration program. They were also given Diamox Sequels, 500 mg nightly, sodium bicarbonate, four 10-grain tablets every 4 hours while awake, and were instructed to test their urines with urine pH test paper to assure their urine pH was ≥ 7.

Tumor parameters consisted of objective measurements of palpable tumor size, measurements of CEA titers, objective measurement of tumor size by appropriate radiographic and scanning techniques, and subjective assessment of bone pain, secondary to bone metastasis.

RESULTS

In Vitro

Figure 1 shows that the uptake of ^{3}H5FU into L1210 leukemia cell RNA is enhanced by pretreatment with various concentrations of methotrexate. The decreases of enhancement in ^{3}H5FU uptake by methotrexate concentrations > 10^{-5} M may be related to pretreatment with high concentrations of methotrexate. Such could result in reduction of the rate of L1210 cell RNA synthesis (4). Note that the uptake of ^{3}H5FU into human bone marrow cells does not change significantly by pretreatment of the bone marrow cells with various concentrations of methotrexate.

In Vivo

Toxicity of methotrexate and 5-FU therapy (table 2).—Note that five patients complained of blurred vision and three of ataxic gait. Myeloid toxicity was quite acceptable and renal toxicity was not observed. Only three courses of chemotherapy required patient hospitalization for parenteral leucovorin. No life-threatening toxicity was noted.

TABLE 2.—Toxicity of intermediate-dose methotrexate and high-dose 5-FU protocol

Toxicity	No. of patients experiencing toxicity
Leukocyte count (cells/mm³)	
0–1500	4
1500–3000	10
3000–5000	6
Platelet count (cells/mm³)	
0–50,000	2
50,000–125,000	5
125,000	11
Hematocrit	
30%	11
30%–40%	9
CNS	
Blurred vision	5
Ataxic gait	3
Nausea and vomiting	4
Mucositis	3
Renal	0
Required hospitalization for leucovorin administration	3
Total No. of patients studied	20

Tumor responses (table 3).—Note that all of the patients with breast cancer were treated extensively with prior chemotherapy combinations or glucocorticoids or antiestrogen therapy. Patients 2, 6, 7, and 13 had not received prior adjuvant therapy but had presented with gross metastatic disease and had failed the prior therapy as noted in table 3. The other patients shown in this table were all failures of the prior treatment when it was given as an adjuvant to their primary modified radical mastectomies. All patients had failed their prior treatment while receiving the prior treatment except for patient 1, who had an interval of 6 months

TABLE 3.—Patients with metastatic breast cancer

Patient No.	Age	Sex	Estrogen receptor	Metastasis	CEA titer	Response to therapy	Duration of response or disease stabilization	Survival from start of treatment	Prior treatment
1	54	F	Negative	Bone	4.3–1.9	Pain relief	4 mos	7+ mos	CMF
2	52	F	ND*	Bone	11.9–6.4	Pain relief (no response)	—	1 mo/died	FACM-P‡
3	55	F	Negative	Bone	ND	Pain relief	7 mos	7+ mos	FAC-T§
4	57	F	Negative	Local	7.4–1.2	50% reduction	3 mos	4+ mos	FAC
5	48	F	Negative	Local	80–14.7	50% reduction	3+ mos	3+ mos	CMF
6	67	F	ND	Bone	203–181	Pain relief	7 mos	8 mos	ACMF-P
7	63	F	Negative	Bone, liver, lung	ND	Mixed response	—	1 mo/died	ACMF-P
8	66	F	Negative	Bone	ND	No response	—	3+ mos	FAC
9	53	F	Positive	Nodes	ND	30% reduction	4 mos	4+ mos	FAC-T
10	47	F	Positive	Lungs, bone	3.3–2.9	50% reduction	3+ mos	3+ mos	FAC-T
11	40	F	Positive	Bone	ND	No response	—	4+ mos	FAC-T
12	53	F	Positive	Bone	4.0–1.1	Pain relief	3+ mos	3+ mos	FAC-T
13	67	F	Positive	Bone, liver†	3.4–2.3	Pain relief	2+ mos	2+ mos	FAC-P

*ND = not done.
†Received only 200 mg/m² of methotrexate.
‡P = prednisone.
§T = tamoxifen.

after CMF was discontinued until new disease was noted. Most patients had received either 5-FU or methotrexate or both of these drugs at the same time and were found to be refractory when the drugs were given in the usual dosage schedules. Three of the 13 (23%) refractory breast cancer patients so treated demonstrated an objective tumor response lasting 3+ months, as measured by a 50% reduction in measurable metastatic disease. Six of thirteen patients (46%) demonstrated a subjective improvement lasting an average of 4.5 months, manifested by the complete reduction of bone pain from metastasis. In addition, three of these patients (Nos. 1, 12, and 13) had serum CEA levels which decreased to normal. The clinical significance of these CEA decreases remains difficult to assess. Patient 9 had a 30% reduction in tumor size and patients 3 and 6 had disease stabilization for 7 months. Patients 2, 8, and 11 (30%) had no significant response to the chemotherapeutic regimen. Patient 7 had a mixed response as manifested by complete disappearance of a pulmonary lesion without change of an abnormal liver imaging.

In seven patients with metastatic adenocarcinoma of the colon, a remarkable five patients demonstrated significant improvement (table 4). Two of seven patients (28%) demonstrated a significant reduction of > 50% in tumors that were metastatic to the lungs. One of these patients also had complete return to normal of an abnormal liver scan. Both of these patients demonstrated a significant reduction in CEA titer. An additional three patients (43%) demonstrated a subjective improvement as manifested by either pain relief, relief of bowel obstruction, or reduction in CEA titer with weight gain. The average duration of the objective responses was 7.5+ months and the average duration of the subjective improvement was 5+ months. The percent of objective response plus laboratory-documented (decrease in CEA) subjective improvement was equal to 71%. Two of seven patients did not respond and these were the two patients that had been treated with either 5-FU or high-dose methotrexate previously.

DISCUSSION

This preliminary study of the sequential combination of intermediate-dose methotrexate followed in 1 hour by high-dose 5-FU has given us pleasing results in breast cancer patients who have been previously treated with and considered refractory to these same agents when given either singly or concomitantly at the usual lower doses. For adjuvant-resistant patients with metastatic adenocarcinoma of the breast, our new treatment scheduling added at least 3–4 months of additional good disease control for some. Other pleasing improvements were obtained for five of seven patients with metastatic adenocarcinoma of the colon. These patients as a group are generally refractory to most chemotherapeutic modalities. We were quite surprised to

TABLE 4.—Patients with metastatic colon cancer

Patient No.	Age	Sex	Metastasis	CEA titer before/after therapy	Response to therapy	Duration of response or disease stabilization	Survival from start of treatment	Prior treatment
1	64	M	Lung, liver	7.5/0.9	>50%	8+ mos	8+ mos	None
2	65	M	Bone, lung	416/20	>50%	7 mos	7+ mos	Radiation therapy
3	47	M	Abdominal cavity	97/10.7	Weight gain, pain relief	6 mos	6+ mos	None
4	54	M	Lung, liver	ND	No response	—	—	5-FU, methyl-CCNU, high-dose methotrexate
5	72	F	Abdominal	164/1	Pain relief, abdominal mass decreased in size 30%	6+ mos	6+ mos	None
6	70	M	Abdominal	1200/470	Weight gain, pain relief	2+ mos	2+ mos	None
7	61	M	Lung, skin	ND	No response	—	3 wks	5-FU

note a significant reduction in CEA titer in most patients treated with this combination and when objective measurements were available, objective tumor responses were noted in two.

The bone marrow toxicity of these two agents when administered in a sequential manner is quite tolerable and most patients were able to be treated at 3- or 4-week intervals. Significant CNS toxic effects occurred in 20% of patients and were manifested by ataxic gait and/or blurred vision. We take these symptoms in part to represent well-known cerebellar and CNS effects of 5-FU. The minimal frequency of severe myeloid toxicity may relate to leucovorin rescue of methotrexate and in part to our laboratory findings showing that pre-incubation of normal human bone marrow cells with methotrexate was not effective in enhancing the incorporation of tritiated 5-FU into human bone marrow RNA, even though it significantly stimulated tritiated 5-FU incorporation into L1210 cell RNA.

Our study represents only the preliminary results of a phase I–II study based on biochemical and laboratory data obtained in the most part from animal and in vitro human bone marrow and L1210 cell studies. The data strongly suggest that, since intermediate-dose methotrexate followed in 1 hour by high-dose 5-FU was effective in refractory and previously treated malignancy, the proper sequencing of these agents may be an important factor for therapeutic response. The data also suggest that a CMF protocol for adjuvant treatment of breast cancer which currently recommends the administration of methotrexate concomitantly with 5-FU may not be the best way to use these two drugs, since escalating the drug doses and giving them sequentially revealed additional improvement in apparently refractory patients. Whether patients refractory to the CMF protocol who responded to our newer protocol did so because of drug sequencing or drug dose escalation or both is not clear from this study. However, this work does suggest that in patients refractory to the CMF protocol or to the FAC adjuvant combination chemotherapy protocol, one may obtain additional objective responses and improvement administering two or one of the same drugs present in a CMF or FAC protocol, but in a different manner.

The data obtained from the seven patients with metastatic adenocarcinoma of the colon leave us with much optimism for the treatment of this disease. As has been pointed out by Moertel, most therapeutic modalities for metastatic adenocarcinoma of the colon leave a lot to be desired and are generally not associated with significant responses. However, in our small group of patients with this disease, we observed unexpected responses. Whether such responses will lead to increased survival is, of course, not yet evaluable. The usefulness of this chemotherapeutic combination in patients with other malignancies remains to be explored.

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Pharmacologic and Toxicologic Evaluation of Thioproline: A Proposed Nontoxic Inducer of Reverse Transformation[1,2]

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SUMMARY

The ability of thioproline to act as a nontoxic inducer of reverse transformation was examined. Thioproline was unable to effect reproducibly morphologic changes characteristic of the reversed transformed state in HeLa, WI-38VA13, C1300 neuroblastoma, and CHO-K_1 cell lines. Reversal of transformed cells with dibutyryl cyclic AMP was, however, readily demonstrated in both the CHO-K_1 and C1300 cell lines. Thioproline produced severe, life-threatening CNS toxicity in both mice and rats at levels far below those previously reported to be without any toxic effects. We conclude that not only is thioproline incapable of producing a reversal of the transformed state in HeLa and other cell lines in vitro, it is also a compound that must be described as a strongly CNS toxic agent.

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Gosalvez et al have recently reported (1,2) that treatment of HeLa cells with thioproline (thiazolidine-4-carboxylic acid; Norgamem) restored contact inhibition in this cell line. He further has stated that thioproline acts via plasma membrane alterations, producing a morphologic change in HeLa cells similar to that obtained by using dibutyryl cyclic AMP in CHO-K_1 cells (2). Thioproline has thus been described as being one of the first of a new series of noncytotoxic anticancer agents. Although thioproline was not active in experimental tumor systems of mice, including L1210 and P388 leukemias, B16 melanoma, Lewis lung cancer, and human xenograft in nude mice, Gosalvez and co-workers have reported clinical efficacy for thioproline in treating epidermoid carcinoma of the head and neck with pulmonary metastases (3).

The present report describes an examination of the efficacy of thioproline in producing a reversal of the transformed state in HeLa and several other established cell lines. In addition, a preliminary toxicologic evaluation of thioproline was done in both mice and rats.

MATERIALS AND METHODS

WI-38VA13, CHO-K_1, and C1300 cell lines were obtained from and maintained according to conditions described by the American Type Culture Collection (Rockville, MD). HeLa cells were a gift from Dr. John Cidlowski, Department of Biochemistry, College of Medicine, University of Vermont (Burlington). All cell lines were grown in media and sera obtained from Grand Island Biological Co (Grand Island, NY). Dibutyryl cyclic AMP, proline, cysteine, and thioproline were obtained from Sigma Chemical Co (St. Louis, MO). L-3,4-Dehydroproline was obtained from Hoffmann-LaRoche (Nutley, NJ) and formaldehyde from J. T. Baker Chemicals (Phillipsburg, NJ).

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Male Swiss Webster mice (22–26 g) and male Sprague-Dawley rats (250–300 g) were purchased from Canadian Breeders (Montreal, Canada). Mice were housed six per cage and rats five per cage in the central animal facilities of the University of Vermont having controlled temperature, relative humidity, and photoperiods. Food and water were permitted ad libitum.

Thioproline was prepared fresh daily in sterile 0.5% Tween 80 at a concentration such that an injection volume of 0.1 ml/10 g of body weight delivered the desired dose. Thioproline was administered as a single ip injection or five daily ip injections. In studies designed to protect mice from the acute toxic effects of thioproline, mice were given ip injections of either sodium methohexital plus sodium pentobarbital or sodium phenobarbital alone. Subsequent injections of either general anesthetic were administered ip as required to maintain adequate anesthesia.

Preliminary toxicologic investigations were also performed using Sprague-Dawley rats. In these studies, thioproline was administered ip to groups of three rats per dose level. In an attempt to define the causative agent of the thioproline acute toxicity, mice were given ip injections of either *N*-formyl cysteine or formaldehyde. Both compounds have been reported to be metabolic products of thioproline (4,5). *N*-Formyl cysteine was synthesized in our laboratory using the method of du Vigneaud et al (6). The product, twice recrystallized and desiccated, had a melting point in agreement with that reported for *N*-formyl cysteine (6).

RESULTS

Cell Culture Experiments

The ability of thioproline to alter rate of growth, viability, and the "transformed" state was examined in WI-38VA13 subline 2RA (a SV40 virus transformed fibroblast derivative of human embryonic lung), HeLa, CHO-K_1 (a transformed subclone of the parental Chinese hamster ovary line), and C1300 neuroblastoma. The WI-38VA13 line was chosen as an example of a well-characterized human transformed cell line. The HeLa line is the line referred to by Gosalvez in his published reports. Both the CHO-K_1 and C1300 cell lines represent control cultures in which a reversal of the transformed state can easily be produced by dibutyryl cAMP as described by Hsie and Puck (7) and Prasad (8).

WI-38VA13.—Using concentrations of thioproline reported (2) to produce reversal of the transformed state in HeLa cells (ie, 10 and 100 μM) we observed no morphologic change in the transformed fibroblast appearance or rate of growth of WI-38VA13 cells. Higher concentrations of thioproline (ie, up to 1 mM) produced a decrease in viability and thereby lowered growth slightly.

HeLa.—Thioproline was completely ineffective in producing a reversal of the transformed state in this cell line. As in WI-38VA13 cells, the 1 mM concentration produced a decreased rate of growth only by producing an increased degree of cell death. Contact inhibition was never observed in the presence or absence of thioproline. In addition, dibutyryl cAMP was ineffective in reversing the transformed state in HeLa cells. This is not an unexpected finding since Johnson et al (9) have previously demonstrated that dibutyryl cAMP is not capable of reversing the transformed state in various cell lines of epithelial cell origin such as HeLa cells.

CHO-K_1.—Because Gosalvez has alluded to the possibility of thioproline acting in a manner similar to that of dibutyryl cyclic AMP, we examined the capacity of thioproline and dibutyryl cAMP to produce a reversal of the transformed state in a cell line where such changes can be readily observed. In over six separate experiments, dibutyryl cAMP (100 μM) consistently produced a change in CHO-K_1 cells from compact, randomly growing, epithelioid-like non-contact-inhibited cells (fig 1A) to stretched, spindle-shaped, fibroblast-like cells exhibiting a distinct tendency to associate with other cells in parallel arrays (fig 1B).

In parallel studies concentrations of either 10, 100, or 500 μM thioproline produced fibroblast-like changes in only two of six experiments. Changes such as those observed in fig 1C were relatively random events on the culture dish and are *not* representative of the majority of cells. Culture plates were independently graded by three investigators who reached similar conclusions. Concentrations of thioproline > 100 μM consistently produced cell death without any accompanying morphologic transformation.

Because CHO-K_1 cells apparently lack the active gene form needed for proline synthesis, this cell line must be maintained in a medium with an unusually high proline concentration (10). Thioproline may, therefore, be producing cell death not as a membrane reorganizer, but as an antimetabolite to proline. To test this possibility, another proline analog, L-3-4-dehydroproline, was investigated for its ability to produce a reversal of CHO-K_1 cells' transformed morphology. Dehydroproline, like thioproline, produced fibroblast-like changes and distinct areas of cellular organization in only 15%–25% of the surface area of CHO-K_1 cultures. This effect

was difficult to reproduce and does not become apparent until 72–96 hours after addition of either thioproline or dehydroproline. Higher concentrations of dehydroproline produced cell death without reversal of the transformed state.

C1300 neuroblastoma.—C1300 neuroblastoma cultures readily undergo "differentiation" in the presence of dibutyryl cyclic AMP (8). This morphologic change was easily reproduced in C1300 cultures grown in our laboratories. Incubation of this cell line with varying concentrations of thioproline up to 1.0 mM, however, produced only a decreased rate of growth via an increased rate of cell death. No change in morphology was ever observed in the C1300 line when grown in the presence of either thioproline or dehydroproline.

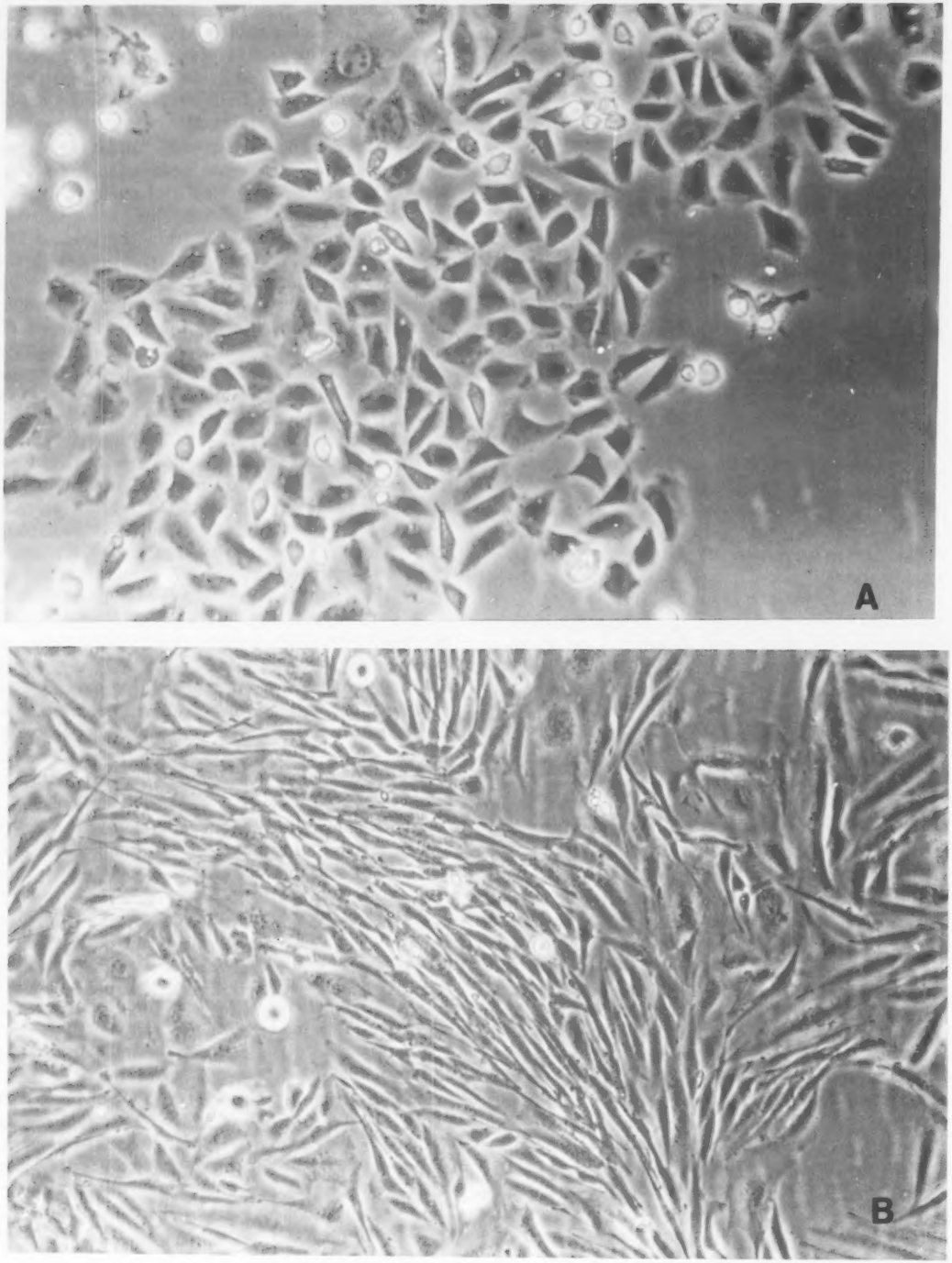
In vivo toxicity experiments.—Mice received a single ip injection of thioproline at doses of 0, 125, 143, 160, 200, 250, or 315 mg/kg (0, 375, 429, 480, 600, 750, or 945 mg/m^2). Within 30 minutes after thioproline administration, acute toxic effects were observed in all thioproline-treated animals. The severity of these toxic signs, which were dose-related, included hyperactivity and increased agitation followed shortly by tonoclonic convulsions. Death resulted within 1 hour of drug administration at doses of $\geq$ 143 mg/kg (429 mg/m^2). All animals which survived the acute neurologic symptoms apparently recovered completely and displayed no further signs of toxicity during the 14-day observation period. However, since histopathologic examination was not performed on any tissues of these animals, one cannot eliminate the possibility that tissue lesions did exist. The calculated LD10, LD50, and LD90 ($\pm$ 1 SE) values for thioproline were 110 $\pm$ 9.5, 164 $\pm$ 4.0, and 246 $\pm$ 13.7 mg/kg (330 $\pm$ 28.5, 492 $\pm$ 12, and 738 $\pm$ 41.1 mg/m^2), respectively (table 1).

Similar toxic effects were observed in mice administered thioproline ip for 5 consecutive days at doses of 0, 80, 100, 125, 143, 160, 200, 250, or 315 mg/kg (0, 240, 300, 375, 429, 480, 600, 750, or 945 mg/m^2). Although the majority of deaths occurred after the initial ip injection, two mice given thioproline at 160 mg/kg (480 mg/m^2) and two mice given thioproline at 143 mg/kg (429 mg/m^2) died following subsequent thioproline treatments. In each case, however, death resulted from the acute neurologic effects of thioproline. At the 125-mg/kg (375 mg/m^2) dose, mice displayed hyperactivity and increased agitation following each thioproline treatment but at no time did the toxic effects progress to convulsions and death. No toxic signs were observed in mice given 80 or 100 mg/kg of thioproline. Again, since no histopathologic examinations were done, there is the possibility that tissue lesions were present. The calculated LD10, LD50, and LD90 $\pm$ values for thioproline given as five daily ip injections were 112 $\pm$ 18.1, 142 $\pm$ 10.6, and 187 $\pm$ 10.5 mg/kg (336 $\pm$ 54.3, 426 $\pm$ 31.8, and 561 $\pm$ 31.5 mg/m^2) respectively (table 2).

Since thioproline toxicity was apparently manifested exclusively as acute neurologic symptoms, it was of interest to determine whether or not barbiturates were capable of controlling these seizures and protecting the animals from the acute lethal effects of thioproline. To test this, mice were given either sodium methohexital (60 mg/kg) plus pentobarbital (60 mg/kg) or phenobarbital alone (100 mg/kg ip). Once anesthetized, mice were given 400 mg/kg (1200 mg/m^2) of thioproline (1.6 times the calculated LD90 of thioproline). The combination of methohexital and pentobarbital, given as needed, during the first hour after thioproline, to maintain general anesthesia, totally eliminated the convulsant activity of thioproline. All six mice that were pretreated with barbiturate survived thioproline treatment, whereas all six mice administered thioproline alone died within 25 minutes of treatment. After phenobarbital (100 mg kg) had produced obvious CNS depression (within 30 minutes), mice were given 400 mg/kg (1200 mg/m^2) of thioproline. Within 15 minutes of thioproline injection, mice displayed convulsant activities not unlike those observed in mice given thioproline alone. The severity of the convulsions was decreased by phenobarbital pretreatment. All six mice pretreated with phenobarbital survived the convulsions and were alive at the end of the 14-day observation period, whereas all six mice given thioproline alone died within 30 minutes of treatment.

Since thioproline is chemically closely related to proline it was of interest to determine whether proline caused similar toxic effects or whether proline could protect mice from the acute toxicity of thioproline. To test this, six mice were given 1000 mg of proline/kg (3000 mg/m^2) ip with no observable toxic effects. Another six mice were given 1000 mg/kg of proline ip 1 hour prior to and immediately after an ip injection of 250 mg of thioproline/kg (750 mg/m^2). All six of these mice displayed characteristic neurotoxic signs and died within 30 minutes of treatment.

A total of nine Sprague-Dawley rats were given thioproline ip at doses of 125, 250, or 500 mg/kg (750, 1500, or 3000 mg/m^2). Acute toxic effects were observed in rats administered either 250 or 500 mg/kg (1500 or 3000 mg/m^2) and included mild excitation followed shortly by mild convulsions. Unlike the toxic signs observed in mice, however, no rat died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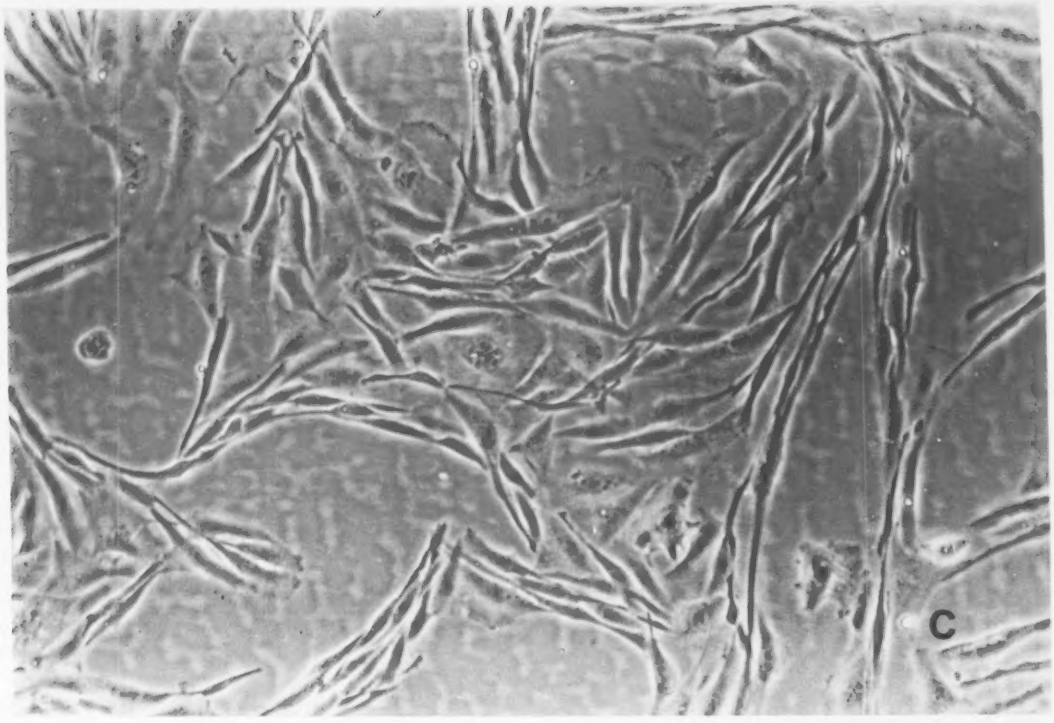

FIGURE 1.—CHO-K_1 cells were plated at 5×10^4 cells/ml in F12 media + 10% fetal calf serum. 4 hrs later, after cells had attached to flask, the media were changed. The cells were then grown for 48 hrs in the presence of media + 10% fetal calf serum as controls (1A), or in the presence of 100 μM dibutyryl cyclic AMP (1B) or 100 μM thioproline (1C). The photographs were taken at 100 × using phase contrast. Incubation conditions included 37°C temperature, 100% relative humidity, and 5% CO_2 atmosphere.

TABLE 1.—Acute toxicity of thioproline given as a single ip injection to Swiss Webster mice*

Dose		No. of mice treated	Deaths†
Mg/kg	Mg/m²		
315	945	6	6
250	750	6	6
200	600	6	4
160	480	6	3
143	429	6	2
125	375	6	0

*All deaths occurred within 1 hr after drug administration.

†LD10 (± 1 SE) = 110 ± 9.5 mg/kg (330 ± 28.5 mg/m²); LD50 (± 1 SE) = 164 ± 4.0 mg/kg (492 ± 12 mg/m²); LD90 (± 1 SE) = 246 ± 13.7 mg/kg (738 ± 41.1 mg/m²).

TABLE 2.—Toxicity of thioproline given as 5 daily ip injections to Swiss Webster mice*

Dose		No. of mice treated	No. of deaths	Day of death†
Mg/kg	Mg/m²			
315	945	6	6	1, 1, 1, 1, 1, 1
250	750	6	6	1, 1, 1, 1, 1, 1
200	600	6	5	1, 1, 1, 1, 1
160	480	6	5	1, 1, 1, 2, 5
143	429	6	3	1, 2, 4
125	375	6	0	
100	300	6	0	
80	240	6	0	

*All deaths occurred within 1 hr after treatment on the day of death recorded.

†LD10 (± 1 SE) = 112 ± 18.1 mg/kg (336 ± 54.3 mg/m²); LD50 (± 1 SE) = 142 ± 10.6 mg/kg (426 ± 31.8 mg/m²); LD90 (± 1 SE) = 187 ± 10.5 mg/kg (561 ± 31.5 mg/m²).

immediately from convulsions; rather, all rats became lethargic shortly after the mild convulsions. Deaths occurred 1–3 days after thioproline injection. Thus, rats responded to ip administration of thioproline in a similar but less severe manner than did mice.

Two different behavioral responses were observed in mice administered either *N*-formyl cysteine or formaldehyde. Mice given 400 mg of *N*-formyl cysteine/kg (1200 mg/m²) became lethargic within 30 minutes after treatment with no significant excitation observed at any time. Death did not occur until 3–5 days after *N*-formyl cysteine treatment. Immediate convulsions and death within 5 minutes were observed in all three mice given formaldehyde ip at a dose of 400 mg/kg (1200 mg/m²). When formaldehyde was administered ip at a lower dose of 200 mg/kg (600 mg/m²), less severe CNS excitation was observed and no deaths occurred. Finally, since thioproline is an analog of cysteine, it was of interest to determine whether or not cysteine could cause similar CNS toxicity. Cysteine was administered as a single ip injection at either 500 or 1000 mg/kg (1500 or 3000 mg/m²) with no observable toxic effects.

DISCUSSION

Thioproline has recently been reported to induce reverse transformation in transformed HeLa cells (1,2). Further, although thioproline was without effect against a panel of experimental murine tumor systems in vivo, Gosalvez et al (2) reported significant clinical efficacy against epidermoid carcinoma of the head and neck with no toxicity at doses as high as 40 mg/kg (1440 mg/m²). Because of the potential importance of these findings, we examined the efficacy of thioproline in producing reverse transformation in several transformed cell lines as

well as determining the toxicologic potential of thioproline in mice and rats.

The results of these studies indicate that thioproline was without effect in producing a reversal of the transformed phenotype of either the HeLa or WI-38VA13 cell lines. These are not unexpected findings, however, since the HeLa cell line has previously been reported by Johnson et al (9) to be unresponsive to dibutyryl cyclic AMP treatment. Two cell lines, the CHO-K_1 and the C1300 neuroblastoma, were both found to be responsive to dibutyryl cAMP as previously reported by several investigators (7,8). These results indicated that both cell lines were capable of undergoing reverse transformation with the proper stimulant. Thioproline, on the other hand, induced reverse transformation in only two of six experiments using the CHO-K_1 cells and never induced reversal of phenotype in the C1300 neuroblastoma. It is important to note that the CHO-K_1 cell line requires proline in the media for proper growth. Since thioproline is an analog of proline, the sporadic morphologic changes may well be related to altered proline metabolism. This possibility is further suggested by the fact that dehydroproline, another proline analog, had similar effects on the CHO-K_1 cell line. It was observed during these studies that isolated areas of apparent reverse transformation occurred in some of the thioproline-treated CHO-K_1 cells. The interpretation and importance of such observations are at present unclear due to the incomplete manner in which this reverse transformation manifests itself in the CHO-K_1 culture and the sporadic nature of its reproducibility.

Although Gosalvez et al (2) have reported thioproline to be nontoxic at doses as high as 40 mg/kg (1440 mg/m²), our studies have shown thioproline to cause acute CNS toxic effects when given ip at doses of ⩾ 125 mg/kg (375 mg/m²) to mice. This toxicity is characterized by severe convulsions and death at doses of 143 mg/kg or more. The toxicity of thioproline to mice is apparently limited to acute effects. This is substantiated by three observations. First, no delayed deaths or toxic signs were observed in mice that survived the acute effects of thioproline. Second, the LD10 and LD50 values of thioproline were not significantly different whether given as a single or as five daily ip injections, suggesting no additive toxicity when multiple injections were given. The LD90 was evidently related to acute toxicity, since mice which were given five daily ip injections of thioproline died only from the immediate toxic effects of thioproline with no delayed toxic effects observed. Finally, mice given an otherwise lethal dose of thioproline (400 mg/kg; 1200 mg/m²) could be protected from the acute CNS toxicity by barbiturate anesthesia and survive with no apparent residual toxic effects. However, since histiopathologic examination was not performed on tissues of these animals, it is possible that treatment-related microscopic lesions did exist.

In preliminary studies we observed that rats responded to thioproline in a similar manner as did mice but were apparently less sensitive to the acute CNS toxicity of thioproline. Further, two putative metabolic products of thioproline (ie, *N*-formyl cysteine and formaldehyde) produced vastly different toxic effects when given ip to mice. *N*-Formyl cysteine at 400 mg/kg (1200 mg/m²) caused lethargy and delayed death (3–5 days after treatment) while formaldehyde at 400 mg/kg (1200 mg/m²) caused immediate convulsions and death within 5 minutes. The levels at which these two agents were administered are probably far greater than that which might be expected to result from the metabolism of similar doses of thioproline. Whether or not *N*-formyl cysteine or formaldehyde actually results from thioproline metabolism in vivo is beyond the scope of the present study. It is important to note, however, that each proposed metabolic product does have the potential for causing either immediate severe CNS toxicity or death.

The pharmacologic and toxicologic properties of a given compound can be affected by the route of administration used. Indeed, Gosalvez (11) in a recent letter has attributed the relative lack of toxicity by thioproline to the fact that thioproline was administered iv in his studies and because of this, is not hydrolyzed in the stomach to cysteine and formaldehyde as suggested by others (5). Garnier et al (5) have reported severe CNS convulsions with status epilepticus and death in accidental ingestion of toxic amounts (at doses as low as 30 mg/kg; 1110 mg/m²) of thioproline by children. They further stated that 60 mg/kg (2220 mg/m²) can cause seizures in adults. The similarity of toxic effects observed by us when thioproline was administered ip, to those described by Garnier et al (5), when thioproline was taken orally, strongly suggests that one should be aware of the convulsant potential if thioproline is to be used in the treatment of solid tumors. In view of our findings and the report of Garnier, it is recommended that further toxicity studies be done in animals before large-scale clinical trials are initiated in the US.

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Early Phase Pharmacokinetics of Doxorubicin (Adriamycin) in Plasma of Cancer Patients During Single- or Multiple-Drug Therapy[1,2]

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SUMMARY

Doxorubicin (Adriamycin [AM]) was measured in plasma in the early distributive phase in 26 patients with different solid tumors. Pharmacokinetic parameters were calculated during first and/or subsequent courses of AM injected iv at doses from 40 to 60 mg/m² either alone or with other antineoplastic agents. A fluorimetric technique was employed to determine AM equivalents; the reduced metabolite Adriamycinol was quantitated by scanning fluorescence after separation in thin-layer chromatography. The following conclusions are drawn from the findings. (*a*) After repeated AM treatments the individual variability in pharmacokinetic parameters of the drug did not appear to be affected by the length of treatment. (*b*) When the same patient was given different AM doses over a certain period some degree of dose dependence was seen only in the area under the curve and extrapolated plasma drug concentration at Time 0. (*c*) A narrow range of AM doses gave pharmacokinetic values differing from one patient to another. (*d*) After repeated AM treatments, Adriamycinol levels became lower, suggesting either a reduced metabolism of the parent compound or increased excretion of the reduced metabolite.

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Doxorubicin (Adriamycin [AM]) is becoming increasingly important because of its broad spectrum of anticancer activity particularly against some solid tumors and hematologic malignancies (1–5). This glycoside antibiotic of the anthracycline group is closely related to daunorubicin (DM), but has superior therapeutic activity (6,7). Its usefulness, however, is limited by the fact that it gives rise to toxic responses such as cardiotoxicity and bone marrow depression (8–15). Since it has been established that plasma levels of AM correlate with its toxicity in patients with abnormal hepatic function (4,16), more knowledge of the clinical pharmacokinetics of AM appears to be needed to improve therapy. This study was undertaken to provide additional information on the pharmacokinetics of AM in plasma of cancer patients receiving intermittent iv therapy. Our intention was to study in detail the early distributive phase. Although this phase usually attracts little attention, it might well be the most important pharmacokinetic determinant of drug response, in view of the rapid tissue uptake of AM in vivo (17,18) and in vitro (19).

The distribution of total AM fluorescence, unchanged drug, and its reduced metabolite Adriamycinol (AMol) was analyzed after single or sequential courses of treatment in order to study the

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extent of intra-individual variability and to investigate whether previous doses affect drug disposal or metabolism. The variability of pharmacokinetic parameters among patients treated with a narrow range of AM doses was determined and differences arising in combined drug therapy were also examined.

MATERIALS AND METHODS

Subjects and Eligibility Criteria

Twenty-six patients at the University of Milan (Hospital L. Sacco and Clinica L. Mangiagalli) were admitted to this study. They included 18 women and eight men, 23–72 years of age. Diagnoses were ovarian epithelial carcinoma (11 cases), cervical carcinoma (four) endometrial adenocarcinoma (one), lung carcinoma (eight), mesothelioma (two). Only patients with normal hepatic, cardiac, and renal functions and hematologic values were admitted to the protocol; they were checked for hepatic and renal function before each AM cycle.

Chemicals and Drug Administration

The following drugs were used clinically: AM (Farmitalia, Milan), methotrexate (MTX) (Cyanamid, Catania), 5-FU (Roche, Milan), cyclophosphamide (CTX) (Asta Werke, Germany), BCNU (Simes, Milan), and hexamethylmelamine (HMM). Pure AM, DM, and AMol as fluorimetric and chromatographic standards were kindly supplied by Prof. Arcamone (Farmitalia, Milan).

AM, alone or in combination with other antitumor agents, was injected iv over about 90 seconds at doses ranging from 20 to 60 mg/m^2, and treatment was repeated at approximately 21- or 28-day intervals according to the various schedules. In polychemotherapy regimens AM was always the first drug given, about 30 minutes before any other agent. If wbc counts were < 2000/mm^3, treatment was discontinued until values returned to well above these limits. Three patients with ovarian cancer and one with cervical carcinoma received AM as a single agent at doses from 40 to 60 mg/m^2; 22 patients received polychemotherapy as shown in table 1.

Drug Assay

Two ml of venous blood was collected and heparinized before the 90-second AM infusion, and at 5, 10, 15, 30, 45, 60, and 75 minutes and 2, 3, and 4 hours after the infusion. The blood samples were immediately centrifuged and plasma was separated and frozen at –20°C until use. Total-drug fluorescence was measured in 26 patients at the first cycle and in three of them at subsequent cycles. The AM metabolite AMol was measured in one patient.

Plasma was extracted and deproteinized with *n*-butanol and samples were analyzed by quantitative spectrofluorimetry in an Aminco Bowman spectrofluorometer according to Finkel et al (20). At 475-nm activation wavelength and 580-nm emission, recovery was 90% and sensitivity was 0.025 μg/ml. Since AM and its metabolites have similar fluorescence spectra, this method does not differentiate accurately between them, so the amounts of AM measured are expressed as AM equivalents.

To separate and quantify AMol, samples of plasma were submitted to thin-layer chromatography and quantified with a fluorescence scanning technique (CAMAG-TLC scanning accessory attached to a Turner model fluorometer Model 111), originally described by Watson and Chan (21) and modified by Benjamin et al (22). After 0.5-ml samples of plasma were extracted once with 2 ml of ice-cold chloroform:isopropanol (1:1), the resulting gel was mixed with sufficient solid ammonium sulfate to give a saturated solution. The organic phase was transferred to a 10-ml Pyrex tube and evaporated under vacuum to dryness. The residues were redissolved with 50 μl of a chloroform:methanol (1:1) solution containing DM as internal standard (25 ng/50 μl). The whole samples were spotted, using a Hamilton microliter syringe, on silica gel plates (DC-Alufolien Kieselgel 60, 20 × 20 cm, 0.2 mm thick; Merck), which were run in an ascending fashion, first for 16 cm in pure ethyl acetate, then for 12 cm in chloroform:methanol:acetic acid: water (80:20:14:6). The plates were dried briefly and allowed to stand for 10 minutes before scanning. An activation wavelength of 475 nm and an emission of 580 nm were used for quantification of AM and AMol. The plasma concentration of each fluorescent spot was determined from the ratio of the fluorescent areas of standard drug solution to the DM area. Recovery was about 70% and sensitivity was ~ 12.5 ng/ml.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated by mathematically analyzing the plasma levels up to 4 hours after treatment by the peeling method (Hewlett-Packard 9810 A), assuming a simple two-compartment open model. The AUC was considered a model independent parameter and was calculated by trapezoidal integration.

RESULTS

The disappearance of AM from plasma was first analyzed in two patients with ovarian carcinoma (Nos. 1 and 2) given repeated courses of AM (table 2). Plasma drug concentrations were monitored by measuring total drug fluorescence from 5 minutes to 4 hours after administration, the aim of the present work being to study early phase pharmacokinetics. The drug dose was adjusted according to the blood cell count; when the wbc count was < 2000/mm^3 treatment was postponed, and the dose was subsequently reduced parallel to the wbc count. The extrapolated Co, half-life, Vd, and AUC are shown in table 2 as representative of the pharmacokinetic parameters calculated for each course of treatment.

Total-drug fluorescence shows a short distribution phase in patient 1 (half-life = 4-7 minutes) followed by a second phase with longer half-life (90-209 minutes). In patient 2 the short distribution phase ranged from 4 to 5 minutes and the second phase from 81 to 157 minutes. Neither α- nor β-half-lives seem to be dose-dependent, whereas Co and AUC appear to be dose-related. These parameters do not seem to be affected by the length of treatment.

For patient 3 (table 3), a patient with carcinoma of the cervix (stage IV) given five courses of AM (40 mg/m^2, iv push), both nonmetabolized AM and its reduced metabolite AMol were measured together, and total-drug fluorescence was determined. The differences in kinetic parameters at subsequent treatments, either for total fluorescence or for AM as such, were again unrelated to the number of cycles. Moreover, the kinetic behavior of AM, as expressed by the parameters considered, paralleled that of the total fluorescence. The reason could be that although metabolites account for part of the fluorescence measured, a large fraction (consistently > 50%) is always represented by unchanged AM, at least during the time investigated (AUC values for AM in the various cycles were always 30%-40% lower than the AUC of the total fluorescence).

The pattern of disappearance of the reduced metabolite AMol is similar to that of AM (fig 1). The drug-disappearance curve shows a biphasic pattern following first-order kinetics. Comparing the percentages of total-drug fluorescence for AM and AMol at different times after administration to patient 3 (table 4), it can be seen that within 4 hours, AM decreases while AMol slowly rises to comparable levels. In this patient, AMol seems to be produced less in the fifth than in the first cycle, the largest amount of AMol found being 55% in the first cycle and 27% in the fifth. Hepatic and renal functions at the first and fifth cycle were within normal ranges as they were in the other two patients (Nos. 1 and 2) who received repeated courses of AM therapy (table 5).

When multiple-drug therapy in concomitant (or sequential) treatments was given to patients with gynecologic or lung tumors (table 1), the broad individual variability in the kinetic parameters of AM made it impossible to assess whether differences ascribable to the drug association occurred. In an attempt to obtain an idea of the extent of inter-individual variability, patients bearing solid tumors who received AM at different doses were divided into three groups according to scalar dose ranges. For each group the highest and lowest values found are indicated in table 6. For a narrow range of AM doses individual variability is striking (eg, Co ranges from 1.4 to 11.7 μg/ml for doses around 40 mg/m^2; this means the coefficient of variability [CV] of the Co measurement is 57% whereas the CV of the administered dose is only 6%), and clearly not related to the systematic variability of the AM measurements. It may be important to note that the broadest variability observed in measurements of AM in plasma of our laboratory animals is 25.2% ± 6.7%, as expressed by the CV.

DISCUSSION

This study presents further information on the pharmacokinetics of AM in human plasma in the early distributive phase. Drug distribution was investigated during first and subsequent courses of AM injected alone or in multiple-drug regimens. AM levels were mostly recorded by measuring total-drug fluorescence and are expressed as AM equivalents; if not otherwise specified, the kinetic parameters discussed in this report also refer to AM equivalents. In our conditions, ie, within the time period investigated (5 minutes to 4 hours), the kinetic parameters for the nonmetabolized compound (except AUC and Co which are direct functions of the amount of compound present) are almost identical to those of total-drug fluorescence (*see* table 3), so that early pharmacokinetics of the latter may well represent the kinetics of AM, at least in the first part of the curve. This parallelism between kinetics of drug equivalents and unchanged AM might arise from our limited observation time, thus explaining the discrepancy between our findings and the report by Benjamin et al (16) of shorter half-life values for the native compound.

In our conditions AM follows first-order kinetics according to a biphasic disappearance from plasma. The calculated α- and β-half-lives are comparable to the short and intermediate half-lives, about 11.6

TABLE 1.—Pharmacokinetic parameters of AM in 1st cycle in 4 patients receiving AM alone and in 22 patients receiving concomitant multiple-drug therapy

Patient No.	Age (yrs)	Diagnosis	Weight (kg)	Previous therapy*	AM dose (mg/m²)	Concomitant therapy (mg/m²)	Pharmacokinetic parameters†				
							Co (μg/ml)	Half-life (mins) α	Half-life (mins) β	Vd (liter)	AUC (μg/ml × min) after 4 hrs
1	23	Ovarian carcinoma	47.1	THBSO, TCT, CTX	60	—	6.3	4	118	181	62
2	61	Ovarian carcinoma	90.5	PR, CTX	60	—	7.2	4	81	176	57
3	72	Squamous cell carcinoma, cervix	42	—	40	—	5.8	7	261	232	59
4	53	Ovarian carcinoma	67	—	50	—	8.1	4	112	112	79
5	32	Ovarian carcinoma	65.5	CTX, PEP	40	CTX, 100, orally, × 14 days	1.4	6	124	265	35
6	48	Ovarian carcinoma	42	PR	48	CTX, 100, orally, × 14 days	5.1	5	223	186	59
7	44	Ovarian carcinoma	80	—	42	CTX, 100, orally, × 14 days	1.7	7	221	294	42
8	46	Ovarian carcinoma	52	PR	46	CTX, 100, orally, × 14 days	4.3	4	156	272	40
9	43	Ovarian carcinoma	54	THBSO	50	CTX, 100, orally, × 14 days	6.4	4	207	235	55
10	57	Undifferentiated carcinoma, lung	59.5	—	40	CTX, 600, iv BCNU, 50, iv } q4wks	2.4	6	214	370	34
11	62	Undifferentiated carcinoma, lung	58	—	40	CTX, 600, iv BCNU, 50, iv } q4wks	3.3	7	219	276	48
12	54	Undifferentiated carcinoma, lung	48	—	40	CTX, 100, iv BCNU, 50, iv } q4wks	6.4	6	198	164	70
13	64	Epidermoid carcinoma (slightly differentiated), lung	66	AM+VCR+CTX	40	CTX, 600, iv BCNU, 50, iv } q4wks	5.0	6	199	211	65
14	60	Mesothelioma	60	TCT	38	CTX, 600, iv BCNU, 50, iv } q4wks	5.8	4	95	259	28
15	66	Undifferentiated carcinoma, lung	62	—	40	CTX, 600, iv BCNU, 50, iv } q4wks	4.4	7	242	160	52
16	63	Mesothelioma	59	—	22	CTX, 600, iv BCNU, 50, iv } q4wks	5.1	4	195	152	39
17	68	Epidermoid carcinoma, lung	58	—	20	CTX, 600, iv BCNU, 50, iv } q4wks	2.3	5	293	295	29
18	52	Squamous cell carcinoma, cervix	58	THBSO, TCT	40	CTX, 100, orally, Days 1–5 5-FU, 400, iv, Days 1–8 } q4wks	3.8	5	180	247	45
19	65	Squamous cell carcinoma, cervix	58	TCT	40	CTX, 100, orally, Days 1–5 5-FU, 400, iv, Days 1–8 } q4wks	7.5	4	138	130	60
20	39	Ovarian carcinoma	70	THBSO	50	CTX, 100, orally, Days 1–4 HMM, 150, orally, Days 1–4 } q4wks	16.8	3	146	138	77
21	45	Ovarian carcinoma	64.5	PR	50	CTX, 100, orally, Days 1–14 HMM, 150, orally, Days 1–14 } q4wks	10.2	3	132	145	58
22	50	Ovarian carcinoma	53	PR	50	CTX, 100, orally, Days 1–14 HMM, 150, orally, Days 1–14 } q4wks	10.1	4	159	105	25

23	41	Squamous cell carcinoma, cervix	59	THBSO, TCT	40	MTX, 20, im, Days 1–8	11.7	3	154	63	149
24	71	Endometrial adenocarcinoma	61	TCT	30	MTX, 20, im, Days 1–8	9.3	5	267	90	85
25	63	Adenocarcinoma, lung	45	Lobectomy	35	MTX, 25, iv, Days 1,7,14,21 5-FU, 300, iv, Days 1,7,14,21 } q4wks	2.7	5	108	252	25
26	64	Adenocarcinoma, lung	50	Lobectomy	37	MTX, 25, iv, Days 1,7,14,21 5-FU, 300, iv, Days 1,7,14,21 } q4wks	4.6	5	166	170	51

*THBSO = total hysterectomy with bilateral salpingo-oophorectomy; TCT = telecobalt therapy; PR = partial surgical removal; PEP = peptichemio; VCR = vincristine.

†Co = extrapolated plasma concentration at time 0; Vd = volume of distribution; AUC = area under the concentration versus time curve. Pharmacokinetic analysis by the peeling method and AUC by trapezoidal integration.

TABLE 2.—Pharmacokinetic parameters of AM equivalents after repeated courses in 2 patients with ovarian carcinoma

Date (mo/day/1977)	AM dose (mg/m^2, iv)	Wbcs ($\times 10^3/mm^3$)	Co ($\mu g/ml$)	Half-life (mins) α	Half-life (mins) β	Vd(1)	AUC ($\mu g/ml \times$ mins) after 4 hrs
Patient 1*							
5/2	60	4.0	6.3	4	118	181	62
5/23	50	3.2	4.8	5	147	215	39
6/14	40	3.0	3.2	5	90	126	48
7/4	—	1.8	—	—	—	—	—
8/21	—	1.6	—	—	—	—	—
8/29	—	2.0	—	—	—	—	—
9/20	60	3.2	6.2	7	126	141	80
10/10	40	2.2	3.0	6	209	240	48
Patient 2†							
4/20	60	4.6	7.2	4	81	176	57
5/19	44	6.2	3.7	5	157	236	52
6/14	36	2.2	3.2	4	132	298	39

*Patient 1 was 23 yrs old, had mucinous adenocarcinoma of the ovary, stage II (FIGO), after THBSO and betathrone, 4700 rads (Aug–Dec 1975); laparoscopy showed metastatic peritoneal spread and liver scan showed dishomogeneous area of distribution in left liver lobus; in June 1976 repeat liver scan was normal; patient treated with CTX (31.5 g) (Apr 1976–Feb 1977), omentectomy, and multiple biopsies (Apr 1977) for minimal residual disease followed by combined treatment with AM, 6 courses (May–Oct 1977). Oct 1977 liver scan gave doubtful result, but liver function had been normal since Apr 1976. 3rd re-staging by laparotomy (Mar 1978) had negative finding. Patient is free from disease: complete response.

†Patient 2 was 61 yrs old, had serous cystoadenocarcinoma of the ovary, stage IIaii, after THBSO and CTX, 44 g (Dec 1975–Mar 1977); omentectomy and multiple biopsies (Mar 1977) for persistent tumor were followed by AM, 3 courses (Apr–Jun 1977). There was rapidly measurable increase of the tumor (peritoneal carcinosis): no response.

TABLE 3.—Pharmacokinetic parameters of AM equivalents measured as total-drug fluorescence (TFL) and of nonmetabolized AM after 5 courses of 40 mg/m^2 (iv push) in patient 3*

Date (mo/day/1977–1978)	Co ($\mu g/ml$)	Half-life (mins) α	Half-life (mins) β	Vd(1)	AUC ($\mu g/ml \times$ mins) after 4 hrs
10/19					
TFL	5.8	5	195	178	59
AM	4.6	4	113	185	28
11/10					
TFL	10.2	4	166	114	79
AM	7.1	5	245	198	65
12/5					
TFL	6.5	5	183	152	67
AM	4.7	5	180	269	41
1/5					
TFL	5.8	6	159	171	56
AM	5.1	5	83	136	37
1/30					
TFL	11.3	2	163	159	38
AM	10.6	2	141	193	26

*Patient 3 was 72 yrs old, had cervical squamous cell carcinoma, stage IV (FIGO), with cancerous vesico-vagino-rectal fistula and left hydronephrosis. Treatment included single-agent AM, 5 courses (Oct 1977–Feb 1978), with a closure of the fistula and 40% reduction of uterine tumor. *See* table 1 for abbreviations.

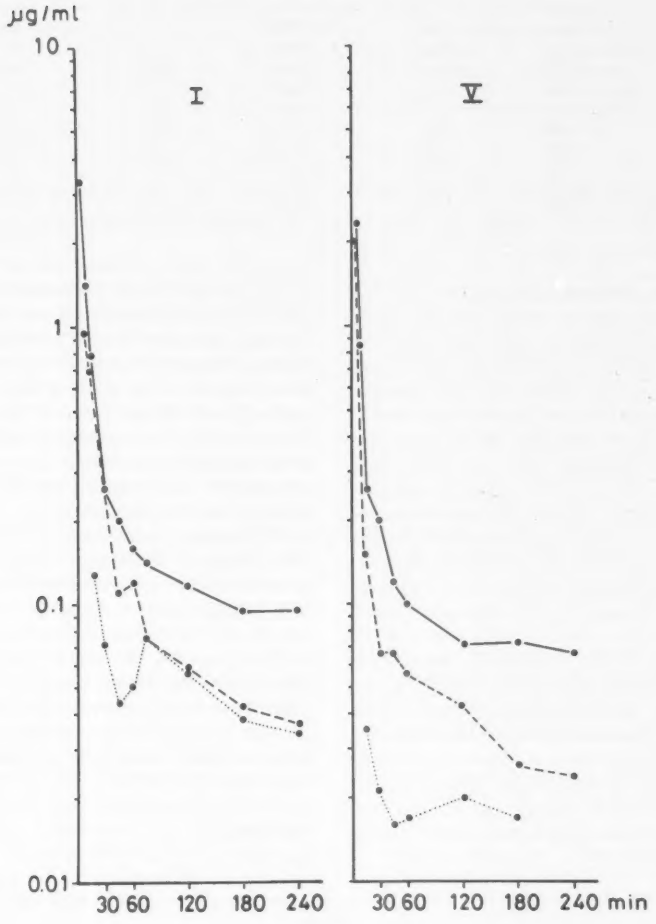

FIGURE 1.—Disappearance of total-drug fluorescence, of nonmetabolized AM, and of its reduced metabolite AMol in plasma in patient 3 after the 1st (I) and 5th (V) cycles of treatment (40 mg/m² iv). ●——● = total fluorescence; ●-----● = AM; ●.....● = AMol.

TABLE 4.—Unchanged AM and AMol in patient 3, during the 1st (I) and 5th (V) cycles of treatment

Mins after treatment	Total fluorescence (μg/ml)		% unchanged AM		% AMol	
	I	V	I	V	I	V
5	3.3	2.3	ND*	86.3	ND	ND
10	1.4	0.8	65.6	70.6	ND	ND
15	0.8	0.2	88.6	58.6	16.6	3.7
30	0.2	0.2	ND	33.0	27.7	10.5
45	0.2	0.12	57.6	55.8	21.7	13.3
60	0.16	0.1	74.5	53.8	31.1	16.3
75	0.14	ND	54.0	ND	55.3	ND
120	0.12	0.07	49.6	59.7	47.8	27.8
180	0.09	0.07	44.2	36.1	40.0	23.6
240	0.09	0.06	38.9	36.4	35.8	ND

*ND = not determined.

minutes and 3 hours, respectively (22), from a triphasic plasma disappearance curve for the same range of doses. This again is a consequence of our sampling schedule limited to early times and ending before the beginning of the third portion of the decay curve described by Benjamin et al (12 hours) for AM, so that the short and long half-lives of our biphasic analysis are actually the short and intermediate half-lives of Benjamin's triphasic calculation. A large discrepancy is seen between our results and those of other investigations (16,18,21,23) if the plasma disappearance curves of AM are analyzed according to a biphasic instead of a triphasic pattern, as already discussed (22). Our very short α-half-life (2–7 minutes), shorter than reported in the literature (18,22,23,24), is probably due to the different initial sampling times (very close in our study) and to a different regression analysis, since in our conditions it was calculated on the drug decline between 5 and 30 minutes as opposed to longer intervals by the other investigators. This rapid initial clearance of AM from plasma can be ascribed either to the rapid tissue uptake mentioned previously (17–19) or to fast metabolism of this compound. The high levels (about 30%) of AMol (22,25) already observed 30 minutes after drug administration help explain the rapid metabolic clearance of the native drug. In accordance with previous reports (21,22) the pattern of disappearance of AMol from plasma appears similar to that of AM; the two compounds are present at approximately equal concentrations between 75 and 240 minutes, at least during the first treatment.

With regard to repeated courses of AM in single-drug therapy, the range of individual variability of pharmacokinetic parameters in the three patients investigated does not appear to be affected by the length of treatment or the doses employed.

That previous treatments may in some way affect the physiologic dispostion of AM and/or its metabolism in the early phase is further suggested by the plasma disappearance curve of AMol. The lower levels of AMol after five treatments (about 50% less than after the first injection) might indicate an impaired carbonyl reduction of AM and an increased metabolism and excretion of AMol. However,

TABLE 5.—Hepatic and renal functions at beginning and end of treatment in patients given AM therapy

Patient No.	No. of AM courses	Total bilirubin (mg/100 ml)	SGOT (unit/liter)	SGPT (unit/liter)	Total nitrogen (mg/100 ml)	Creatinine (mg/100 ml)
			*Beginning of treatment**			
1	6	0.6	13	15	26	1
2	3	0.7	14	16	27	1.1
3	5	0.7	19	15	20	1.1
			*End of treatment**			
1	6	0.5	19	17	27	1.1
2	3	0.6	10	7	22	1.1
3	5	0.8	25	10	23	1.1

*The following were considered normal values: total bilirubin < 1.2 mg/100 ml; SGOT and SGPT < 25 unit/liter; total nitrogen < 40 mg/100 ml; creatinine < 1.2 mg/100 ml.

TABLE 6.—Pharmacokinetic parameters of scalar doses of AM in patients with different solid tumors

No. of patients	Dose range (mg/m^2)	Pharmacokinetic parameters*				
		Co ($\mu g/ml$)	Half-life (mins)		Vd(1)	AUC ($\mu g/ml \times$ mins) after 4 hrs
			α	β		
3	20–30	5.6 (2.3–9.3)	4.7 (4–5)	252 (195–293)	179 (90–295)	51 (29–85)
15	35–46	4.6 (1.4–11.7)	5.5 (3–7)	178 (95–261)	224 (63–370)	54 (25–149)
8	48–60	8.8 (6.3–16.8)	3.9 (2–5)	147 (81–223)	160 (105–235)	59 (25–79)

*Range is shown in parentheses.

whether this result observed in only one patient is representative of a more general situation obviously has to be confirmed.

When AM is given in multiple-drug regimens with other antitumor agents, CTX, MTX, 5-FU and BCNU, its kinetic parameters are the same as when it is given alone. However, there is no way of ascertaining whether these compounds interfere with the distribution of AM because of the high variability observed and the limited number of patients treated with AM alone. Chang et al (26) reported that combined treatment with a nitrosourea derivative other than BCNU, streptozotocin, affects AM disposition by increasing drug exposure. The kinetic parameters obtained in our patients show striking inter-individual variability for equal AM doses. This broad difference in AM disposition in different individuals is again in good agreement with reports by other investigators (22, 27-29). In our conditions the differences in where patients were treated, in the methods of estimating dosages, and in how rapid the injection was could contribute to the variability found.

It is questionable whether the differences in pharmacokinetics of AM in different patients help predict the clinical response to this compound, in other words, whether drug levels are correlated to therapeutic effects. Further studies are warranted to clarify the reasons for this variability and to investigate whether AM disposal is reflected in clinical response.

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Preclinical Toxicologic Evaluation of 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (AMSA) in Mice, Dogs, and Monkeys[1,2]

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SUMMARY

Male mice (CDF_1) in a single-treatment schedule, male and female dogs in a single-treatment and in three multiple-treatment schedules, and male and female monkeys in one multiple-treatment schedule were used to evaluate the toxicity of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide. Vehicle controls receiving anhydrous *N,N*-dimethylacetamide plus lactic acid were included in the dog and monkey studies. The liver was the major target organ of toxicity in mice. Lymphoid depletion and generalized bone marrow suppression were the most sensitive indicators of toxicity in dogs and monkeys. At high toxic doses, CNS and intestinal toxic effects were present. Hepatotoxic effects were most prominent in dogs after single iv injections. Multiple-dose regimens were more toxic than single-dose regimens in dogs, but inclusion of rest periods between multiple-treatment periods or reduction of the dose attenuated the toxic effects. The monkey was more resistant to drug toxicity than the dog. Local tissue reaction studies in guinea pigs and rabbits suggested that local irritation responses observed may have been due to the acidity of the drug and vehicle solutions.

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The experimental antineoplastic compound 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (AMSA) is highly active in the L1210 screening system and appears schedule-dependent, being more effective if dosed repeatedly at short intervals. Use of this agent resulted in long-term survivors in the B16 malignant melanoma and P388 leukemia assays (1), but was ineffective against a mouse ependymoblastoma (2). After iv or ip injection of the labeled compound, radioactivity was rapidly eliminated in the bile, with more than one half of the administered radioactivity excreted in the first 2 hours (3). Plasma ^{14}C levels declined biphasically, with an early rapid component followed by a much more gradual decline; levels were relatively higher in liver, bladder, and spleen than in other tissues examined. Effects on the immune system included almost complete suppression of the immune response (4).

Phase I clinical studies of AMSA have been performed, and hematopoietic toxicity was observed (5,6). Myelosuppression, and to a lesser extent, thrombocytopenia, were the predominant toxic responses and dose-limiting factors. This paper presents the results of the preclinical toxicologic studies of AMSA in mice, dogs, and monkeys.

MATERIALS AND METHODS

Experiments were performed on CDF_1 mice, bea-

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gle dogs, and rhesus monkeys in accordance with the procedures outlined by Prieur et al (5) and modified by the Battelle Columbus Laboratories Toxicology Program Office.[6] Dogs were supplied by the Mammalian Genetics and Animal Production Section (Bethesda, MD), through the Battelle Toxicology Program Office, and monkeys were purchased from Primate Imports. These animals were housed in individual steel cages and provided with water ad libitum. Fecal examinations for parasites were performed for dogs and monkeys prior to the study. The monkeys were held in isolation for a minimum of 6 weeks and were tuberculin-negative three consecutive times before they were released for treatment. At least two complete hematologic and serum chemistry determinations were done on each dog and monkey before treatment. After drug treatment the parameters that were analyzed included physical examinations, hematologic and serum chemistry determinations, ophthalmic examinations, and urinalyses, followed by necropsies and histopathologic evaluations. The femur was the source of bone marrow for both sections and smears (M:E ratios). Lymph nodes examined included mesenteric, mandibular, bronchial, and regional, ie, near the injection site. The thymus and tonsils were also evaluated. Both dogs and monkeys were treated in pairs, one male and one female at each dosage level. The treatment schedules used in this study are shown in table 1.

Male and female CDF_1 mice were supplied by the Mammalian Genetics and Animal Production Section (Bethesda, MD). They were housed in polycarbonate cages containing hard wood bedding. Purina mouse chow and water were available ad libitum. Single iv doses (tail vein) were administered to 13 animals of each sex (20 g of body weight). Only ten males and ten females per dose level were used in the calculation of the median lethal dose (LD50); the remaining three animals per sex served as a satellite group. If an animal that was used for the LD50 determination died unobserved, the most severely affected animal from the satellite group was killed and necropsied at that time for histopathologic evaluation.

Hartley strain guinea pigs, approximately 200–300 g, and New Zealand rabbits, approximately 1.8–2.2 kg, received sc or im injections, respectively, on single and five daily dose schedules to test for local tissue reaction. Two guinea pigs or one rabbit per regimen were used. The maximum concentration of compound used was the toxic dose high (TDH) established in the single-dose study in dogs. This

[6]Procedures Manual. Battelle Columbus Laboratories, Feb 5, 1975.

TABLE 1.—Treatment schedules and dose ranges used for toxicologic evaluation of AMSA in CDF_1 mice, beagle dogs, and rhesus monkeys

Species	Treatment schedule	Dose range (mg/m^2)
Mouse	Single injection	78.90–114.00
Dog	Single injection	15.62–1000.00
	Daily × 5 days	1.96–31.24
	Daily on Days 1–5, 15–19, and 29–33, with rest periods on Days 6–14 and 20–28	1.96–7.80
	Daily × 10 days	0.98–7.80
Monkey	Daily × 5 days	3.90–124.80

was dissolved in 5 ml of vehicle; one-half and one-quarter dilutions of this solution and the vehicle were also injected. Constant volumes (0.5 ml) of the vehicle and drug solutions were injected into guinea pigs. Rabbits received 0.5 ml of the three dilutions or the highest concentration in volumes of 0.5, 0.25, and 0.125 ml. The vehicle was injected in equivalent volumes. Blood compatibility of AMSA and the vehicle were tested with canine blood, serum, and plasma.

The formulation for AMSA consisted of two sterile liquids that were aseptically combined prior to use. The drug solution contained 1.5 ml of a 50-mg/ml solution of AMSA in anhydrous *N,N*-dimethylacetamide (DMA). The drug-DMA solution was added to a vial containing 13.5 ml of 0.0353 M L-lactic acid to produce an orange-red solution containing 5 mg/ml of AMSA, 2.8 mg/ml of L-lactic acid, and 100 mg/ml of DMA, pH 3.5. Vehicle solutions contained equivalent amounts of DMA and L-lactic acid, pH 3.2. Drug solutions were prepared immediately before iv treatment.

The following definitions of dose levels were used in this study: *highest nontoxic dose (HNTD)*—the highest dose at which no hematologic, chemical, clinical, or morphologic drug-induced alterations occur; doubling this dose produces the aforementioned alterations; *toxic dose low (TDL)*—the lowest dose which produces drug-induced pathologic alterations in hematologic, chemical, clinical, or morphologic parameters; doubling this dose produces no lethality; *TDH*—the dose which produces drug-induced pathologic alterations in hematologic, chemical, clinical, or morphologic parameters; doubling this dose produces lethality; *LD*—the lowest dose which produces drug-induced death in any animal during the treatment or observation period.

RESULTS

An orange precipitate was present in mixtures

of AMSA and canine serum or plasma at drug concentrations of 1.0 mg/ml (1 vol of 5 mg/ml drug and 4 vol of serum/plasma). The vehicle was compatible with serum-plasma. The drug produced hemolysis at concentrations of ≥ 0.556 mg/ml; the vehicle produced hemolysis at a ratio of 1:1 with whole blood.

Local tissue reaction studies in guinea pigs and rabbits suggested that the acidity of the drug solutions and vehicle may have been responsible for the local tissue reaction and irritation responses. The defined toxicity levels in mice, dogs, and monkeys are shown in table 2.

Median Lethal Dose in Mice

Dose-response lethality curves were determined for AMSA, DMA-lactic acid (vehicle), and DMA. The maximum volume of L-lactic acid that could be given by iv push (3.0 ml) did not produce lethality or histopathologic lesions. The 14-day LD50s for male and female CDF_1 mice after single iv injections of AMSA were, respectively, 34.4 and 33.0 mg/kg (103.2 and 99.0 mg/m²). All deaths occurred within 4 hrs of treatment. Toxic signs at LDs included hypoactivity, prostration, dyspnea, and convulsions immediately preceding death. Maximum weight loss was observed 3–8 days after treatment. Tissue changes in the liver were present in mice dying on Day 1 and in those killed on Day 14. The severity of the lesions (swelling and vacuolation of hepatocytes in both centrilobular and periportal areas) was positively correlated with dosage level. The LD50 for male and female mice collectively for the vehicle, expressed as amount of DMA per kilogram, was 2341 mg/kg (7023 mg/m²). Solutions of DMA in water resulted in a collective LD50 for males and females of 2680 mg/kg (8040 mg/m²). The LD50s of AMSA for males and females contained DMA doses of 688 and 660 mg/kg of DMA, respectively. Thus, much larger doses of vehicle were required to produce lethality in mice than were administered in the drug-vehicle toxicity studies. Histologic lesions in mice receiving the vehicle or DMA were similar to those found in drug-treated mice.

Single-Dose Study in Dogs

Eight pairs of dogs were used in this study. One pair was used as a vehicle control and seven pairs were injected iv with single doses of 50, 25, 12.5, 6.25, 3.125, 1.562, or 0.78 mg/kg (1000, 500, 250, 125, 62.5, 31.25, or 16.52 mg/m²). The controls received an amount of vehicle equivalent to the milliliter per kilogram administered at a drug dose of 12.5 mg/kg. Four lethal doses were injected in this study and the survival rate was correlated with dosage level. At a 50-mg/kg dose the dogs died within 10 mins or 48 hrs; at a 25-mg/kg dose they died on Day 5; and after treatment with a 12.5-mg/kg dose they died on Day 5 or Day 6. One dog treated with a dose of 6.25 mg/kg was killed on Day 8, the other animal died on Day 10. Only the dog that died immediately after treatment with the highest dosage level showed clinical signs of nervous system effects: tremors, ataxia, and tonic convulsions. Anorexia, adipsia, emesis, hematochezia, miosis, dyspnea, and weight loss were observed at LDs. Pyrexia and copious salivation occurred at a dose of 6.25 mg/kg. Toxic signs after treatment with

TABLE 2.—Defined toxicity levels of AMSA in mice, dogs, and monkeys

Species	Study	Toxicity	Dose	
			mg/kg	mg/m²
Mouse	LD50	LD90	37.300	111.90
		LD50	33.700	101.10
		LD10	30.400	91.20
Dog	Single dose	LD	6.250	125.00
		TDH	3.125	62.60
		TDL	1.562	31.24
		HNTD	0.780	15.62
Dog	5 daily doses	LD	0.780	15.60
		TDH	0.390	7.80
		TDL	0.196	3.90
		HNTD	0.098	1.96
Monkey	5 daily doses	LD	10.400	124.80
		TDH	5.200	62.40
		TDL	0.650	7.80
		HNTD	0.325	3.90

nonlethal doses or the vehicle were confined to reduced food consumption.

At the two highest dosage levels, hemoconcentration, leukocytosis followed by leukopenia, increased BUN, creatinine, alkaline phosphatase (AP), SGPT, bilirubin, magnesium, and decreased calcium levels occurred. At 12.5- and 6.25-mg/kg dose levels, hemoconcentration, leukocytosis, and changes in renal function tests were not observed. Serum levels of AP, SGOT, SGPT, and sulfobromophthalein (BSP) retention were increased. Severe leukopenia was also observed with slight to moderate depression of platelet counts. At the 3.125-mg/kg dose level, depressions of rbc and wbc counts and increased SGOT and SGPT levels were observed. At lower doses, changes were limited to decreased wbc and rbc numbers. Vehicle controls had marked increases in AP, SGOT, and SGPT levels.

Tissue changes observed appeared to be dose-related. Generalized congestion and hemorrhage, lymphoid depletion, bone marrow depression with almost complete depletion of hematopoietic elements, and vacuolation and necrosis of hepatocytes were present. Dogs treated with the 6.25-mg/kg dose also had severe peripheral necrosis of the palatine tonsil with bacterial invasion and colonization. Severe bacterial pneumonia (lobar, with colonization, necrosis, alveolar edema and hemorrhage) and perivascular edema were observed in lung sections from these dogs. At lower doses, lesions included mild congestion of intestinal mucosa, mild to moderate lymphoid depletion, focal hemorrhages and necrosis in lymphoid tissue, mild to moderate bone marrow depression, and swelling of centrilobular hepatocytes. Treatment with the vehicle produced similar lesions: mild congestion of ileal and cecal mucosa, mild lymphoid depletion, and focal hemorrhage in lymphoid tissue.

Five Daily Dose Study in Dogs

Eight pairs of dogs were used in this study. Two pairs were used as vehicle controls (DMA-lactic acid), one pair was used as a saline control, and five pairs were injected iv with five consecutive daily doses of 1.56, 0.78, 0.39, 0.196, or 0.098 mg/kg (31.42, 15.62, 7.8, 3.9, or 1.96 mg/m^2). Vehicle controls received an amount of vehicle equivalent to that administered at doses of 1.56 and 0.78 mg/kg. Saline controls received volumes similar to that given with the highest dosage level of drug. The two highest dosage levels were lethal in this study. The dogs treated with 1.56 mg/kg were killed in extremis on Day 6. At the 0.78-mg/kg dose level, one dog was necropsied on Day 6 and the other dog, moribund, was killed on Day 9. Toxic effects at these doses were similar to those seen after single doses; stiff gait, congestion of ocular blood vessels, and hyperthermia were also evident. A reduction in food consumption occurred at the 0.196-mg/kg dose level. Clinical signs were absent at lower doses and in the control pairs.

Changes in hematologic and serum chemistry values were most prominent shortly before death and were similar to those seen after single doses. Marginal increases in SGPT levels and moderate increases in AP were observed. Neutropenia and anemia, as evidenced by a decrease in hematocrit, hemoglobin, and rbc count, occurred after treatment with both lethal doses. The only change in hematologic or serum chemistry values at lower dosage levels was a decreased total wbc count which was present at the 0.39-mg/kg dose level.

Severe generalized bone marrow depression occurred in dogs treated with doses of $\geq$ 0.39 mg/kg. Lymphoid depletion, necrosis, focal hemorrhage, erythrophagocytosis, and hemosiderosis were most prominent at higher doses and in the dogs killed on Day 6. Slight effects on lymphoid tissue were observed at the 0.196- and 0.098-mg/kg dose levels. Severe mucosal lesions (diffuse hemorrhage and congestion) were evident in the small intestine and colon of dogs that were given the two highest doses. Swelling and vacuolation of centrilobular hepatocytes were observed at nonlethal doses and were most prominent in dogs observed for 45 days after treatment. Lesions in the control pairs occurred only in the one dog treated with the smallest vehicle volume. Splenic lymphoid follicle hypoplasia was evident in this dog.

Five Daily Dose Study in Monkeys

Eleven pairs of monkeys were used in this study. Four pairs were used as vehicle controls, one pair was used as a saline control, and six pairs were injected iv with five consecutive daily doses of 10.4, 5.2, 2.6, 1.3, 0.65, or 0.325 mg/kg (124.8, 62.4, 31.2, 15.6, 7.8, or 3.9 mg/m^2). Vehicle controls received volumes equivalent to those administered at doses of 10.4 mg/kg and twice this volume, and at doses of 5.2 mg/kg and one half of this volume. A pair of monkeys were injected with five consecutive doses of 0.25 ml/kg of saline. One monkey treated with the highest dose level was killed on Day 6; the other monkey died on Day 9. Emesis, diarrhea, anorexia, miosis, hyperpnea, and excessive salivation occurred at the 10.4-mg/kg dose level. Manifestations of clinical signs were absent at lower doses.

A moderate decrease in the hematocrit, hemoglobin, and rbc values was observed at the highest dose level. Neutrocytosis occurred during treatment and a marked lymphopenia was present after the end of treatment. Marginal increases in SGOT occurred on Days 4 and 6. At the 5.2-mg/kg dose level, increased neutrophil counts occurred during treatment; leukopenia and anemia were observed after treatment. Similar but less severe changes were observed at the 2.6-mg/kg dose level. Lymphopenia was evident through the 45-day period and depressions of total wbc and platelet counts occurred on Day 52. Monkeys treated with lower doses had slight to moderate depressions of wbc count which occurred 1–2 weeks after treatment. Changes in hematologic and serum chemistry values in controls were not remarkable.

Severe generalized bone marrow depression occurred in monkeys treated with doses of ≥ 2.6 mg/kg. Slight to moderate depletion of bone marrow cells was observed at the two lowest dose levels. Focal necrosis and hypoplasia of lymphoid tissue were evident at all doses; the severity of the lesions was dose-related. Diffuse, generalized swelling of hepatocytes was observed at the ≥ 1.3-mg/kg dose level. Tissue lesions were reduced or absent in monkeys held for observation. Cloudy swelling of hepatocytes was observed in two vehicle control monkeys: one that received the largest volume of vehicle and the other that received the lowest volume.

Multiple-Dose Study With Rest Periods in Dogs

Three pairs of dogs were used in this study. The treatment schedule was five consecutive daily injections for three treatment periods with intervening 9-day rest periods. Dose levels used were the defined TDH, TDL, and HNTD from the 5 daily dose study in dogs: 0.39, 0.196, or 0.098 mg/kg (7.8, 3.9, or 1.96 mg/m^2). Reduced food consumption or anorexia was the clinical sign of toxicity and occurred only at the highest dose during the first treatment period.

Slight depression of rbc counts occurred at the two highest dose levels during the three treatment periods. Leukopenia, neutropenia, and a moderate depression of platelet count were present at the highest dose; a slight decrease in platelet count occurred in one dog after treatment with 0.196 mg/kg. Changes in liver function tests were observed only during the third period. Slight to moderate increases in BSP retention and SGPT levels were evident at the 0.39-mg/kg dose level and a slight increase in BSP retention was evident in one dog treated with 0.196 mg/kg. At the lowest dosage level one dog gave indications of severe hepatotoxicity, with marked increases in BSP retention, SGPT, SGOT, and AP levels observed during the third treatment period.

Tissue changes were most prominent in the dogs killed 24 hrs after the end of treatment. Focal necrosis and lymphoid depletion, bone marrow depression with a predominant myelopoiesis, and swelling of hepatocytes occurred in dogs at the two highest dose levels. Mild tissue lesions were present in dogs at the 0.098-mg/kg dose level.

Ten Daily Dose Study in Dogs

Four pairs of dogs were used in this study. The treatment schedule was ten consecutive daily injections. Daily dose levels were one half the defined doses from the five daily dose study in dogs: 0.39, 0.196, 0.098, or 0.049 mg/kg (7.8, 3.9, 1.96, or 0.98 mg/m^2). Food consumption was reduced in the two dogs treated at the highest dose during the last few days of treatment. Changes in hematologic values occurred after the sixth injection. A slight decrease in rbc count in both dogs and leukopenia with a depression of platelet count in the female was observed at the 0.39-mg/kg dose level. Slight to marginal decreases in rbc and wbc counts occurred at lower doses. Severe generalized bone marrow depression occurred only at the highest doses in the dog killed on Day 11. Lymphoid depletion was evident at all doses and its severity was correlated with dose level. Diffuse cloudy swelling of hepatocytes was observed in dogs killed 45 days after treatment.

DISCUSSION

Toxic changes in the intestinal tract, lymphoid tissue, bone marrow, liver, kidneys, and CNS occurred after treatment with AMSA. Toxicity to the CNS was observed at lethal doses in mice and dogs and was manifested by tremors, ataxia, and tonic convulsions. Emesis, anorexia, diarrhea, hematochezia, and microscopic observations of congestion and hemorrhage in the mucosa were signs of gastrointestinal toxicity of lethal doses in dogs and monkeys. Changes in renal function tests were also associated with the administration of lethal doses and occurred in conjunction with hemoconcentration and changes in serum ion levels. The major target organs for drug toxicity at nonlethal doses were the liver, lymphoid tissue, and bone marrow.

Generalized bone marrow depression and lymphoid depletion occurred in all treatment regimens.

These changes could generally be correlated with depression of the total wbc count. Leukopenia has been previously reported in phase I clinical trials as the major dose-limiting factor (3,4). Neutropenia was present in dogs at the higher dose levels. Lymphopenia was more prominent in monkeys than in dogs. Hematocrit, hemoglobin, and rbc values were decreased from baseline values in most dogs treated at nonlethal doses. The depression in platelet count was not as severe as reductions in other circulating blood cells and was not a consistent finding. Mild thrombocytopenia has also been observed in clinical studies (3,4).

Hepatotoxicity was demonstrated in dogs by increased serum chemistry values, AP, SGOT, SGPT, bilirubin, and BSP retention, and microscopic evidence of hepatocellular necrosis, vacuolation, and swelling. The increases in serum chemistry values and tissue lesions were most severe after single iv injections. Liver toxicity was not a major clinical sign in the multiple-dose regimens. Onset of hepatotoxicity was delayed in the multiple-dose study with rest periods in dogs and individual animal susceptibility was observed since one dog treated with the lowest dose showed marked increases in serum chemistry values. Smaller daily doses of AMSA on a ten consecutive daily dose schedule had no effect on liver function tests. Microscopic liver lesions in dogs receiving the multiple-dose regimens were confined to diffuse cloudy swelling of hepatocytes.

Increases in liver function tests that occurred after iv injection of the large single doses of the vehicle suggested that DMA may contribute to the hepatotoxic lesions present in drug-treated animals. Previous studies with DMA showed that it caused elevations in serum enzymes diagnostic for hepatocellular damage (6). In the rodent LD50 study, liver lesions were present in both drug- and vehicle-treated animals. Changes in serum liver function tests or microscopic lesions, however, did not occur when smaller daily volumes of the vehicle were injected (five daily dose study in dogs). Hematopoietic toxicity was not seen in the present study following administration of DMA to dogs and monkeys.

The response in monkeys was similar to that described for dogs. Lymphoid depletion and bone marrow suppression were the most sensitive indicators of toxicity. Neutrocytosis occurred at higher dose levels during treatment; leukopenia and anemia were delayed. Hepatotoxicity was not a prominent feature of AMSA toxicity in monkeys. Marginal increases in SGOT were observed only at the lethal dose. Swollen hepatocytes were observed in monkeys at the $\geq$ 1.3-mg/kg dose level, and in two of the four vehicle-treated monkeys (one that received the largest volume of vehicle and one that received the lowest volume).

A steep dose-lethality curve was obtained in mice and survival time was not dose-related. In dogs, survival was inversely correlated with dose level: the larger the dose the shorter the lifespan. A multiple-dose schedule of five daily injections was more toxic to dogs than a schedule of a single rapid injection. Drug toxicity was reduced, however, when rest periods were interposed between multiple-dose treatment periods or a smaller daily dose was administered in ten injections. On a five daily dose regimen, the monkey was less sensitive to the toxic effects of AMSA than the dog. In the monkey, daily doses of 5.2 mg/kg were tolerated without lethality whereas the TDH in dogs was 0.39 mg/kg. The results of the mouse studies and multiple-dose regimens indicate that the compound is rapidly eliminated, as suggested by earlier studies (3). The severe bacterial pneumonia observed in dogs that received 6.25 mg/kg as a single dose was probably the cause of death. The marked depression of wbc counts in these dogs suggests that AMSA has a strong immunosuppressive effect that may compromise the defense mechanisms against bacterial invasion.

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Phase I Study of a New Antitumor Drug, 1-Hexylcarbamoyl-5-fluorouracil (HCFU), Administered Orally: An HCFU Clinical Study Group Report[1]

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SUMMARY

A phase I study of a new fluorinated pyrimidine, 1-hexylcarbamoyl-5-fluorouracil (HCFU), was performed by a multi-institutional clinical study group using a total of 111 patients with histologically proven malignancies. The characteristic toxic effects were a transient hot sensation and pollakiuria, which occurred 15–120 minutes after oral administration of the drug, continued for 30 minutes to 4 hours, and subsided spontaneously. Gastrointestinal disturbances such as nausea, vomiting, diarrhea, and anorexia, which are common with 5-FU administration, also occurred with HCFU but did so less frequently. The maximum tolerated dose for a single oral administration was estimated to be between 12 and 15 mg/kg and the optimal daily dose for continuous administration was considered to be between 9 and 18 mg/kg, with divided daily administration. Fifty-seven patients received 5–19 mg/kg/day of HCFU for > 4 weeks, including 31 patients with > 60 days' treatment. Cumulative doses were from 9.5 to 166.2 g, with a mean of 26.3 g. Hematopoietic toxicity was slight and hepatic toxicity was questionable. No renal or other cumulative toxicity was observed. In ten of the 57 patients, favorable clinical effects were seen: an active decrease in the size of the solid tumor (three patients), the disappearance of ascites (six), and the improvement of intestinal obstruction due to peritoneal carcinomatosis (one).

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A new fluorinated pyrimidine, 1-hexylcarbamoyl-5-fluorouracil (HCFU) was developed by Hoshi et al (1,2) in 1975–1976 as a masked compound of 5-FU for oral use. As shown in figure 1, the molecular weight is about twice that of 5-FU, and larger than that of ftorafur (FH). Thus, the 5-FU moiety in 100 mg of HCFU is approximately 50 mg.

The superiority of the antitumor activity of HCFU was suggested by several animal experiments (2) which revealed that the therapeutic ratio

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[2]This study was conducted by the HCFU Clinical Study Group (Yoshiyuki Koyama, Chairman). The following institutions and investigators participated in the study: National Cancer Center Hospital (*Yasuo Koyama, Hisanobu Niitani, Keiichi Maruyama, Kenzo Okabayashi, Nobuo Okazaki, Kintomo Takakura, Teruo Sakano,* and *Toru Ise*); National Medical Center Hospital (*Yoshiyuki Koyama, Tadashi Kimura, Yukio Imamura, Osamu Nakajima, Noritsugu Umeda,* and *Ryu-ichi Sakagami*); Cancer Institute and Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (*Tatuo Saito, Isao Nakao, Takao Kanko, Yasuhiko Oh-hashi, Makoto Ogawa,* and *Yukoh Inoue*); Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University (*Masakazu Yokoyama*); Faculty of Medicine, University of Tokyo (*Satoru Kusama* and *Jiro Tomiyama*); Tokyo Metropolitan Komagome Hospital (*Ichiji Itoh, Yasunobu Sakai, Akira Kubo, Takeshi Tominaga,* and *Jun-ichi Ishiwata*); Tokyo Metropolitan Ebara Hospital (*Yoshio Hara*); National Defense Medical College Hospital (*Osamu Takatani*); Aichi Cancer Center Hospital (*Kazuo Ohta* and *Minoru Murakami*); Research Institute for Microbial Disease, Osaka University (*Tetsuo Taguchi, Yosuke Nakano,* and *Kozo Jikuya*); and Faculty of Medicine, Kyushu University (*Kiyoshi Inoguchi, Ryunosuke Kumashiro,* and *Ryuichiro Tamada*).

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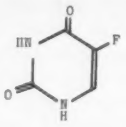
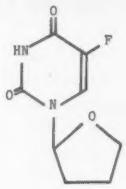
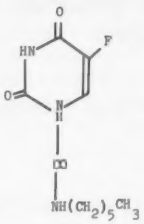
Compound	5-Fluorouracil	1-(2-Tetrahydrofuryl)-5-fluorouracil	1-Hexylcarbamoyl-5-fluorouracil
Abbreviation	5-FU	FH	HCFU
Chemical structure			
Molecular formula	$C_4H_3FN_2O_2$	$C_8H_9FN_2O_3$	$C_{11}H_{16}FN_3O_3$
Molecular weight	130.08	200.17	257.26
M.W. Ratio (approx)	1	1.5	2
5-FU moiety in 100 mg	100 mg	approx. 65 mg	approx. 50 mg

FIGURE 1.—Chemical structures of HCFU, 5-FU, and FH.

of HCFU (4.5) for L1210 tumors in mice was higher than those of FH (1.0) and 5-FU (1.9), and that HCFU also had a broader antitumor spectrum than the other two drugs after oral administration.

Preclinical studies in mice (oral administration) showed LD50 values of 1260 mg/kg for HCFU, 1510 mg/kg for FH, and 332 mg/kg for 5-FU.[3] Principal autopsy findings were pulmonary congestion, gastric hemorrhage, and atrophied thymus, which all three analogs produced. The LD50 values in rats were 268 mg/kg in males and 920 mg/kg in females for HCFU, 1150 mg/kg in males and 1400 mg/kg in females for FH, and 285 mg/kg in males for 5-FU.[3] Those values in mongrel dogs were 65 mg/kg in males and 97 mg/kg in females for HCFU, 48 mg/kg in males for FH, and 22 mg/kg in males for 5-FU.[3] The representative findings in dogs that died were pulmonary congestion and gastrointestinal hemorrhage, which were not essentially different from those in mice and rats; these responses were also produced by all three chemicals.

Subacute and chronic toxicologic studies in rats showed minimal or slight hematologic, hepatic, and renal toxicity (3). Subacute toxicologic studies in beagle dogs showed that death occurred in several animals as early as Days 7–12 of the experiment in each of the groups treated by daily administration of 10 mg/kg of HCFU, 15 mg/kg of FH, and 5 mg/kg of 5-FU (4). The early deaths were usually preceded by convulsions in animals treated with 5-FU and FH. With HCFU, death occurred so suddenly in apparently healthy animals that the cause of death seemed to be different from that with the other two chemicals. However, the principal findings on autopsy of the rats (extended pulmonary edema with congestion and congestive changes of the liver) were common to HCFU, FH, and 5-FU. No particular changes that could be specifically connected with HCFU were found. On the other hand, an irreversible edema around the column of the fornix was found in beagle dogs that were treated with 10 mg/kg/day of HCFU for 6 months (4). Similar but more marked changes in the columnar region of the hypothalamus were also found in animals given the same dose of FH.

In summary, in experimental animals toxicity of HCFU is analogous to that of 5-FU and FH in quality, and its degree is between that of the latter two chemicals in small animals and approximately equal to that of FH in beagles. The early sudden death of beagle dogs treated by moderate doses of HCFU suggested that a dose schedule for the phase I study should be determined prudently, with special attention to a possible correlation with CNS toxicity. The results reported here are those of the phase I study performed by a clinical research group in-

[3]Mitsushima T. Personal communication. Institute of Biological Science, Mitsui Pharmaceuticals, Inc.

volving most of the major medical institutions in Japan.

MATERIALS AND METHODS

Patients.—Patients with histologically proven malignancies refractory to prior therapy who were not eligible for other conventional treatment were admitted to the study. They were required to have a life expectancy of > 1 month, a wbc count of > 3000/mm^3, a platelet count of > 1 × 10^5/mm^3, a normal liver function, and a normal BUN. As exceptions in the cases of liver metastases, patients with < 1.5 mg/ml of bilirubin, < 100 milliunits/ml of SGOT (normal range, < 35 milliunits), < 100 milliunits/ml of SGPT (normal, < 30 milliunits),< 800 milliunits/ml of LDH (normal, 180–380 milliunits), and < 30 units/ml of alkaline phosphatase (normal, 2.6–10.0 units) were also included. The primary sites of malignancies in the 111 patients included in the study were the stomach (40 cases), colorectum (22), breast (18), liver (ten), lung (eight), uterine cervix (four), and pancreas, larynx, esophagus, and brain (one each). One case of neuroblastoma and four cases of metastatic cancer of unknown origin were included. The anticancer drug histories of the patients were: none in 52 patients, 5-FU in 14, FH in 25, and other drugs including mitomycin C, doxorubicin, and cyclophosphamide in 20. The average age of the patients was 53.6 years. There were 60 males and 51 females.

Drugs.—HCFU was supplied in 50- and 100-mg tablets from Mitsui Pharmaceuticals, Inc, Tokyo, Japan. In order to adjust the dose according to body weight, some patients received granules with the same composition as the tablets. It was confirmed by animal experiments that the bioavailability of HCFU was equivalent for both forms of the drug.

Initial dose and schedule of drug administration.—A starting dose for man was determined to be n = 1 mg/kg, calculated from the maximum tolerated dose, the toxic dose low, and the LD50 values for several animals. In the initial phase of the study, a 2-week course of administration was set, because of the results of the subacute toxicologic study in beagle dogs. HCFU was administered orally once a day, in the early morning, for 2 weeks. This course was followed by ≥ 1 week without the drug. After the discontinuation period, some patients in whom no adverse effect was observed were given another course at a higher dose. A schedule for the second stage was determined in accordance with the progress of the study in order to confirm the cumulative toxicity of HCFU. The duration of treatment was prolonged to > 4 weeks when a patient tolerated the 2-week treatment well, with no objective evidence of adverse effect. The single daily dose was divided into two or three daily doses to mitigate the acute adverse responses of a hot sensation and pollakiuria, as described in the Results section. The time of administration was also changed from before to after meals for the convenience of the patients on long-term daily administration, because it was confirmed by absorption-excretion studies in animals and patients that the total bioavailability was scarcely influenced by meals.[4,5] Patients were observed closely and carefully during and after the treatment for any evidence of toxic or other biologic effects, especially on neuromuscular functions. The monitoring of total protein, albumin, bilirubin, SGOT, SGPT, alkaline phosphatase, BUN, and circulating blood cell count with differential count was done weekly during the course of treatment. Informed consent was obtained from the patients before therapy was started.

Evaluation of antitumor effects.—The results of animal experiments (1,2,5,6) suggested that to attain an antitumor effect in man, a certain period of oral HCFU administration might be necessary. Accordingly, antitumor effects were evaluated in patients who received the treatment course for > 4 weeks. The effects were evaluated for measurable lesions: ie, those with a diameter that could be measured repeatedly on roentgenograms or by inspection and/or palpation. In patients with extrahepatic measurable lesions, a positive response was defined as a ≥ 50% decrease in the sum of the products of the maximum perpendicular diameters of the lesions, disease progression was defined as a > 25% increase, and no change was defined as a < 50% decrease. In patients with scan-positive hepatomegalia > 5 cm from the costal margin or xiphoid process, a positive response was defined as a decrease of ≥ 30% in the sum of the measurements at both midclavicular lines and the xiphoid.

RESULTS

Toxicity and Maximum Tolerated Dose in 2 Weeks of Treatment

Thirty-eight of the 111 patients were treated on a schedule of one daily drug dose for 2 weeks. Five of the 38 patients were re-treated at a higher dose level after 2 weeks of treatment and 1 week of observation were completed. Consequently, 43 courses

[4]Sakai Y. Personal communication. Tokyo Metropolitan Komagome Hospital.

[5]Yokoyama M. Personal communication. Institute of Biological Science, Mitsui Pharmaceuticals, Inc.

TABLE 1.—Toxicity from 2-wk treatment

HCFU dose (n)*	No. of evaluable courses	No. of patients with—		
		Pollakiuria syndrome†	Nausea or vomiting	Other toxic effects
1	3	0	0	0
2	2	0	0	0
3	3	0	0	0
5	12‡	2	0	0
7	12§	3	4 (3)	Anorexia: 1 Liver dysfunction: 1
9	6¶	2	1	0
12	2	2 (1)**	0	Desquamation of skin of fingers: 1 (1)
15	1	1	0	0
18	1	1 (1)	0	Fever, anorexia, and liver dysfunction: 1
21	1	1 (1)	0	Anorexia: 1

*n = 1 mg/kg.
†Hot sensation, pollakiuria, and frequent defecation (*see* text).
‡In 1 case, patient was pretreated with 3 n.
§In 2 cases, patients were pretreated with 3 and 5 n, respectively.
¶In 2 cases, patients were pretreated with 5 and 7 n, respectively.
**Nos. in parentheses = No. of patients who had treatment discontinued because of toxic effects.

of treatment became evaluable. The symptoms and signs observed during the 2-week treatment are summarized in table 1 according to dose level. At dose levels up to 3 n no toxic response was found. However, at dose levels of > 5 n, peculiar symptoms not expected from the toxicologic study in animals were observed. The symptoms consisted of a hot sensation around the face, trunk, and/or extremities; pollakiuria without polyuria; and/or urgency to defecate or tenesmus or diarrhea. Some patients receiving higher doses also experienced general fatigue and/or anorexia. Pollakiuria and the hot sensation occurred 15–120 minutes after the administration of HCFU, continued for 30 minutes to 4 hours, and subsided spontaneously. The severity and duration of these symptoms depended on the dose of HCFU and in part on the individual. The details are given in the following case reports.

CASE REPORTS

Patient 1.—Patient 1 is a 45-year-old female with recurrent breast cancer who complained of urgency to urinate 30 minutes after the administration of a 12-n dose of HCFU on the first day of treatment. At the same time she felt general fatigue which was accompanied by a hot sensation around her face. No flushing of the face was seen. Vital signs including pulse rate, respiration rate, blood pressure, and body temperature were essentially unchanged. She had to urinate three times in 30 minutes but passed only a small amount of urine each time. Urinalysis of each fraction was normal. The symptoms continued for 4–6 hours and disappeared spontaneously. The same transient symptoms were seen every day after HCFU administration, and on the fourth day of treatment she refused to take the drug. After discontinuation of the treatment, she never complained of the same symptoms and no significant change in the laboratory data was found during the next 2 weeks.

Patient 2.—Patient 2 is a 44-year-old male with lung cancer who received 18 n of HCFU at 9 AM. At 11 AM, 2 hours after HCFU administration, a hot sensation occurred and continued for about 3 hours. He complained of an urgency to defecate and an episode of dizziness at 1 PM. He evacuated soft stools three times in 30 minutes. The hot sensation and urgency to defecate continued about 4 hours and disappeared spontaneously. An increase in body temperature to 39°C the next day was accompanied by general fatigue and anorexia, and the treatment was discontinued. The fever, general fatigue, and anorexia continued until the third day; however, by the morning of the fourth day all symptoms and signs had disappeared. A transient rise of SGOT and LDH values, from 44 and 650 milliunits to 101 and 1120 milliunits, respectively, was observed in tests on the 21st day but had normalized before the 36th day. Other laboratory findings including circulating blood cell count, differential count, urinalysis, BUN, and creatinine were essentially unchanged during the same period.

Patient 3.—Patient 3 is a 48-year-old male with

adenocarcinoma of unknown origin, metastatic to cervical nodes, who complained of a hot sensation 30 minutes after taking 21 n of HCFU. The hot sensation was followed by pollakiuria and tenesmus. He also complained of general fatigue, a dizzy sensation, and anorexia, which continued for about 10 hours. No rise in body temperature was observed. All vital signs were stable throughout the episode. He slept well with the aid of 10 mg of nitrazepam and although the symptoms had disappeared by the next morning, the treatment was discontinued. Laboratory findings remained unchanged for the next 3 weeks.

The observations described above revealed that the dose-limiting factor of HCFU was a new type of syndrome which is represented by a hot sensation, pollakiuria, and frequent defecation. At higher dose levels the syndrome was so uncomfortable that every patient who was given ≥ 15 n of HCFU strongly refused to take the drug again. Consequently, the maximum tolerated dose for a single oral administration was estimated to be between 12 and 15 n. Another trait of this syndrome was its immediate appearance in contrast to the nausea and vomiting (the second major toxic effect) which appeared at a later stage of the treatment in most of the patients with this type of reaction. Absence of the latter toxic effect at higher dose levels may be due to discontinuance of the treatment in an earlier stage. A slight-to-mild liver dysfunction was observed in two patients and was reversible in both. A transient rise in body temperature to 39°C was seen in only one patient, and its causal relationship to HCFU was not certain. No hematologic, renal, CNS, or peripheral neuromuscular toxic effects, other than the syndrome described above, were found throughout the 43 courses of treatment.

Effect of Divided Administration on Incidence of Toxic Effects

The effect of divided administration on the occurrence of toxic effects was analyzed (with special reference to the pollakiuria syndrome) in a series of 169 trials in 111 patients. The trials were performed one time in 70 patients, two times in 29, three times in five, four times in two, and six times in three. The daily dose levels were grouped into three ranges: 1–7 n, 8–15 n, and > 16 n. A single-dose administration was given 65 times and a divided dose was given 104 times, at various daily doses. The incidence of the pollakiuria syndrome is shown in figure 2; it appeared to be dose-dependent and was clearly decreased by divided administration.

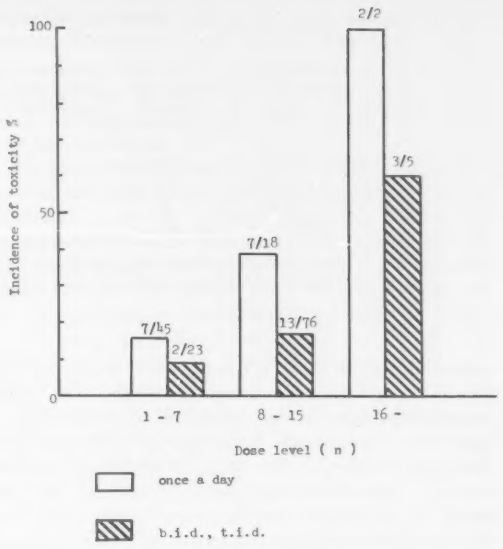


FIGURE 2.—Effect of divided administration of HCFU on incidence of the pollakiuria syndrome. Nos. above bars = No. of trials with toxic effects/No. of evaluable trials.

Cumulative Toxicity

Seventy-three patients were on an administration schedule of > 4 weeks in order to study cumulative toxicity. The daily doses were 5–19 n, which were divided into two or three daily administrations when continuation of the treatment seemed to be difficult because of pollakiuria and/or the hot sensation.

The treatment was continued as long as possible when there was no apparent danger to the patient. Sixteen of the 73 patients were excluded from the evaluation. Of these 16 patients, six could not continue oral administration for > 4 weeks because of aggravation of the primary disease and the other ten were still in the study but the duration of their treatment was < 4 weeks at the time of evaluation. Consequently, there were 57 evaluable patients.

Cumulative doses were from 9.5 to 166.2 g, with a mean of 26.3 g. Thirty-one of the 57 patients were given HCFU for > 60 days. One patient received the maximum daily dose of 16 n twice daily for 65 days (45.5 g), and the longest continuous administration was 12 n three times daily for 277 days (166.2 g).

Toxic effects observed in 57 patients are summarized in table 2 according to the cumulative doses. The degree of the toxic effects did not differ

TABLE 2.—Toxic effects from treatment for > 4 wks

HCFU cumulative dose (g)	No. of evaluable patients	No. of patients with—			
		Pollakiuria syndrome*	Nausea or vomiting	Anorexia	Other toxic effects
≤ 10	6	3	0	0	0
11–30	28	6	2 (1)†	2 (1)	Skin rash: 2 (1)
31–50	18	7	4	3	Alopecia: 1
> 50	5	0	1	0	0
Total	57	16	7	5	3

*Hot sensation, pollakiuria, and frequent defecation (*see* text).
†Nos. in parentheses = No. of patients who had treatment discontinued because of toxicity.

essentially from that in the patients receiving the 2-week treatment. No symptom or sign that could be considered to have a correlation with cumulative dose was recognized. Although special attention was paid to the condition of the CNS and peripheral nervous system of the patients throughout the study, no abnormal condition was found other than the transient irritable syndrome of the autonomic nervous system described above.

The maximum decreases in wbc, rbc, and platelet counts were $3.2 \times 10^3/mm^3$, $267 \times 10^4/mm^3$, and $5.6 \times 10^4/mm^3$, respectively. In 31 patients who were treated for > 60 days, the mean values of wbc, rbc, and platelet counts were $7.7 \times 10^3/mm^3$, $382 \times 10^4/mm^3$, and $23 \times 10^4/mm^3$, respectively, at the start of treatment. At the end of the eighth week of continuous administration the values were $7.3 \times 10^3/mm^3$ for wbcs, $353 \times 10^4/mm^3$ for rbcs, and 24.3×10^4 for platelets, respectively: ie, –5.2%, –7.6%, and +5.6% changes from the initial values. However, the only change that was statistically significant was that for rbcs ($P < 0.01$).

At the beginning of treatment, 35 patients had normal serum biochemistry values and 22 had slightly to moderately abnormal values. Of the 22 patients, 14 had primary or metastatic liver tumors, and eight had suspected metastatic liver disease. Serum biochemistry values moved to the abnormal range during the course of treatment in three of the 35 patients who had normal liver function at the start of treatment. By the time of evaluation, metastatic liver disease had become apparent in all three patients.

On the other hand, abnormal serum biochemistry values returned to normal in four of the 22 patients who initially had had certain or suspected neoplastic involvement of the liver. In the other 18 patients, changes in biochemistry values varied from case to case, essentially reflecting the condition of the malignant disease; no significant change attributable to HCFU was found.

Antitumor Effects

Nineteen of the 57 patients who were evaluated for toxic effects had measurable lesions. Of the 19 patients, three showed a positive response, 13 showed no change, and three showed disease progression. Two patients with a positive response were refractory to previous FH treatment. Among 38 patients without measurable lesions, seven had favorable responses and ten had disease progression. The favorable responses were disappearance of cytology-positive ascites (six patients) and improvement of intestinal obstruction due to peritoneal carcinomatosis (one). All of the favorable responses were accompanied by improvement of performance status and/or by increase in body weight. Two patients showing favorable responses were refractory to previous FH treatment. The other 21 patients showed neither definite improvement nor disease progression.

DISCUSSION

The antitumor activity of HCFU against experimental murine tumors had been observed not only in rapidly growing tumor systems such as leukemia, ascites sarcoma, and solid tumors (1,2), but also in early or advanced slow-growing tumors such as Lewis lung carcinoma, B16 melanoma (5), and spontaneous mammary adenocarcinoma (6). In the latter three systems, the drugs were administered until death of the animal in order to test the possibility of long-term administration. The experimental results indicated that HCFU was more effective than 5-FU and FH for the prolongation of life and also suppressed lung metastases in mice with spontaneous mammary adenocarcinoma (6), which is known to be refractory to conventional antitumor agents.

Subacute and chronic toxicity studies in rats (3) and beagle dogs (4) suggested the possibility of long-term administration in man with less toxicity,

because no specific and serious toxicity due to HCFU as compared with 5-FU and FH was observed.

In the present phase I study, a hot sensation, pollakiuria, and frequent defecation were confirmed as the specific and frequent toxic effects. Those symptoms are thought to be specific to HCFU because they have never been found after oral administration of 5-FU or FH. Furthermore, they are subjective symptoms, and recognition of them depended fully on complaints of the patients. In most cases, the symptoms occurred 15–120 minutes after administration and continued for 30 minutes to 4 hours, with spontaneous disappearance. Most of the patients complained of these transient symptoms after every administration; however, some stopped their complaints in the course of continuous administration. Only a very few patients noticed these symptoms in the later period of continuous administration. It was also confirmed that incidence of these symptoms could be decreased by divided administration. These data suggest that the symptoms may be induced by some unknown mechanism which is specific to HCFU and different from mechanisms of toxic effects such as bone marrow depression and gastrointestinal disturbances which are known to be common to 5-FU and its derivatives. The symptoms characteristic of HCFU could not be predicted from tests in various animals at the time of the preclinical studies. A search for substance(s) which might induce these symptoms had been conducted.

According to a detailed investigation of metabolism, carboxypentylcarbamoyl-5-fluorouracil (CPEFU) and carboxypropylcarbamoyl-5-fluorouracil are found as intermediate metabolites of the conversion of HCFU to 5-FU (7). Recently, both metabolites were confirmed in the blood of patients who received HCFU (8). Furthermore, it was demonstrated in an ongoing study that one metabolite, CPEFU, had a strong detrusor contractile activity when administered iv to rabbits and cats; details of this study will be presented in the near future.

Gastrointestinal disturbances such as nausea, vomiting, and diarrhea were essentially the same as those observed with 5-FU or FH. Throughout the phase I study, however, the incidence of gastrointestinal disturbances was less frequent with HCFU than with the two analogs, ie, the incidence with various cumulative doses was between 9% and 12% with long-term administration of HCFU, but it was 38.6% with FH given orally (9) and 56.5% with 5-FU granules (10). A reversible hepatic dysfunction was seen in two of 43 courses of the 2-week treatment but not in 57 patients who were treated for > 4 weeks; the hepatotoxicity of HCFU in man was not confirmed.

The maximum tolerated dose of HCFU for a single oral administration was estimated to be between 12 and 15 n. In the patients receiving HCFU for a longer time (> 60 days), no serious toxic effects (eg, hematologic, hepatic, renal, or CNS) attributable to cumulation of HCFU has been seen as yet. An absorption-excretion study of HCFU was done during the phase I study and the results will be reported separately. From the viewpoint of toxicity, HCFU may be used for a long time at daily dose levels of 9–18 n with divided administration. Definite antitumor effects were observed in three of the 19 patients who had measurable lesions and received HCFU daily for > 4 weeks.

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Doxorubicin and Ifosfamide Combination Chemotherapy in Previously Treated Acute Leukemia in Adults: A Southwest Oncology Group Pilot Study[1,2]

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SUMMARY

The Southwest Oncology Group did a limited institutional pilot study of the combination of doxorubicin and ifosfamide in the treatment of previously treated adult patients with acute leukemia. Thirty-four patients received one or two courses of the combination. All patients had received prior chemotherapy and 32 had received prior anthracycline chemotherapy. Three patients died before their responses could be fully evaluated. Fourteen patients achieved complete remission (41%) and one patient achieved partial remission. The complete remission rate was 27% for patients with acute myeloblastic leukemia (myelomonoblastic leukemia, monoblastic leukemia, and erythroleukemia) and 89% for patients with acute lymphocytic and undifferentiated leukemias (ALL). Toxic effects included severe hematologic reactions in 33 of 34 patients, hematuria in six patients, altered sensorium in one patient, and congestive heart failure in one patient. The safety of the combination was established and toxic side effects of this therapy were tolerable. The 89% complete remission rate for previously treated patients with ALL suggests that the combination of doxorubicin and ifosfamide may be particularly effective in ALL.

[Cancer Treat Rep 64:869–872, 1980]

Doxorubicin (Adriamycin) and ifosfamide are active as single agents in the treatment of acute leukemia (1–3). When doxorubicin was used as a single agent in previously treated patients with acute leukemia, a complete response rate of 21% was observed (2). Ifosfamide is a cyclophosphamide analog that is only slightly myelosuppressive in doses with effective antitumor activity. Bladder toxicity, the most serious toxic effect of ifosfamide, may be controlled with the simultaneous oral administration of ascorbic acid (4) or with administration of ifosfamide by means of a continuous infusion (5). There is evidence of synergism between cyclophosphamide and anthracyclines in animal tumors (6). Doxorubicin and cyclophosphamide have been shown to be an effective combination (7). Since ifosfamide is minimally myelosuppressive, its combination with doxorubicin allows full doses of each drug to be given.

As reported in this paper, the Southwest Oncology Group did a limited institutional pilot study of the combination of doxorubicin and ifosfamide in the treatment of acute leukemia in adults.

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MATERIALS AND METHODS

Patients

Adult patients with acute leukemia (all cell types) who had had relapses of their disease after prior therapy or who had failed to respond to other therapy were eligible for this study. Patients were required to have > 30% blast cells in the bone marrow. Prior anthracycline therapy was allowed if the total cumulative dose did not exceed 300 mg/m² of doxorubicin or 2000 mg/m² of rubidazone. Patients with any of the following characteristics were ineligible: (*a*) > 75 years old, (*b*) congestive heart failure (CHF), (*c*) BUN > 30 mg/100 ml or creatinine > 2 mg/100 ml, and (*d*) > 10 rbcs/high power field centrifuged urine sediment or currently active urinary tract infection.

Treatment

Doxorubicin (60 mg/m²) was given iv over 20–30 minutes on Day 1. Ifosfamide (1000 mg/m²/day) was given by continuous infusion on Days 1–5 in 3 liters of fluid/24 hours. While receiving ifosfamide therapy, each patient was given 500 mg of ascorbic acid orally every 6 hours and a daily urinalysis was done. If microscopic hematuria exceeded 5 rbcs/high power field, ifosfamide was discontinued for 24 hours or until hematuria cleared. For patients who did not achieve complete remission (CR) of their disease with one course of chemotherapy, re-treatment was allowed on Day 15 if the marrow was hypercellular, on Days 18–21 if the marrow was normocellular, and on Days 21–28 if the marrow was severely hypocellular. Doses of both drugs were reduced by 25% for the second course if the marrow was hypocellular on Day 21. Doses of both drugs were increased by 33% for the second course if the marrow was hypercellular or normocellular before Day 21. Cardiotoxicity due to doxorubicin was assessed by means of electrocardiogram (summed QRS voltage in the limb leads) taken before each course of therapy (8). A 30% reduction in summed QRS voltage necessitated discontinuing doxorubicin therapy. CR, partial response (PR), and failure of response were defined by Southwest Oncology Group criteria (9). Patients who achieved CR received maintenance therapy chosen by their primary physician.

RESULTS

Thirty-four patients, 26 males (76%) and eight females (24%), met the criteria for eligibility and were treated with the combination of doxorubicin and ifosfamide in the previously described manner. The ages of the patients ranged from 12 to 75 years; the median age was 37 years. All patients had received previous chemotherapy for acute leukemia, and 32 patients (94%) had received prior therapy that included an anthracycline derivative, either doxorubicin or rubidazone.

Six patients had failed to achieve CR while receiving chemotherapy, and 28 patients had relapses of their disease after CR. Nine of the 34 patients were classified as having ALL, which in this paper includes acute lymphocytic and undifferentiated leukemias. Twenty-five patients were classified as having AML, which in this paper includes acute myeloblastic leukemia, myelomonoblastic leukemia, monoblastic leukemia, and erythroleukemia.

Of the 34 patients, three died (on Days 7, 14, and 16) before their responses could be fully evaluated: one died of bacterial sepsis, one died of CNS hemorrhage related to thrombocytopenia, and one died of organ failure from advanced leukemia. Sixteen of the 31 remaining patients did not respond to therapy. Fourteen of the 16 nonresponding patients received two courses of chemotherapy, and the remaining two patients each received one course of chemotherapy. One patient achieved a PR as a result of treatment with the combination of doxorubicin and ifosfamide. Fourteen patients achieved CR. Response rates are summarized in table 1.

Of nine patients, eight (89%) with ALL achieved CR. Of 25 patients with AML, six (24%) achieved CR and one achieved PR. Table 2 shows response rates according to cell type. The two patients with ALL who had received no prior anthracycline therapy achieved CR. Four of the six patients who earlier were nonresponders to chemotherapy achieved CR. The prior chemotherapy had included an anthracycline in all six patients. Of the six patients, one died on Day 7 of therapy and one failed to respond.

Duration of response ranged from 3 weeks (in a patient who died of hepatitis type B while in CR) to 116+ weeks. Two patients remain alive and in remission at 19 and 116 weeks. Median duration of CR was 12 weeks. In six patients, duration of CR

TABLE 1.—Overall response rates

Response	No. of patients	% of patients
CR	14	41
PR	1	3
No response	16	47
Not evaluated	3*	9
Total	34	100

*Patients died before response could be fully evaluated.

TABLE 2.—Response rates according to cell type of acute leukemia

Cell type	No. of patients achieving—			No. of patients not evaluated*
	CR	PR	No response	
ALL	8	0	1	0
AML	6	1	15	3

*Patients died before response could be fully evaluated.

was > 6 months. Maintenance chemotherapy after CR was chosen by the patient's primary physician. One patient received maintenance chemotherapy that did not contain doxorubicin (6-mercaptopurine, ifosfamide, and methotrexate). All other patients received maintenance chemotherapy with reduced doses of doxorubicin and ifosfamide.

Hematologic toxicity with this combination of drugs was expected, and 33 of the 34 patients had severe hematologic toxic effects, with platelet counts of < 30,000/mm³ and absolute neutrophil counts of < 500/mm³ during therapy. One patient with neutropenia died of gram-negative sepsis. One patient had mild toxicity with a platelet count nadir of 119,000/mm³ and an absolute granulocyte count nadir of 1480/mm³. Microscopic hematuria occurred in six patients and it ceased when ifosfamide therapy was discontinued. No patient had gross hematuria. One patient had a transient elevation of BUN, which reached a peak at 93 mg/100 ml, but the problem spontaneously resolved. One patient became lethargic and irritable and had altered sensorium and personality change, all of which might have been drug-related.

One 17-year-old boy had CHF. He had received a total dose of 767 mg/m² of rubidazone in a previous study, and he received a total dose of 292 mg/m² of doxorubicin in this study. The CHF was attributed to anthracycline cardiotoxicity.

DISCUSSION

The safety of the combination of doxorubicin and ifosfamide has been established. Hematologic toxicity was expected, but the investigators expressed the subjective opinion that hematologic toxicity with this combination is milder in terms of duration and degree than that usually seen during remission induction of acute leukemia. Bladder toxicity from ifosfamide with microscopic hematuria occurred but was easily managed by discontinuing the ifosfamide therapy. The paucity of problems related to bladder toxicity may be due to the administration of oral ascorbic acid, assuring adequate urine output, and the technique of continuous infusion of ifosfamide. The one episode of renal failure, which spontaneously resolved, is attributed to ifosfamide. The development of doxorubicin cardiotoxicity is dose-related in most instances, and difficulties in determining safe dose levels with the various anthracycline analogs may have contributed to the development of this toxic effect in one patient.

The CR rate for previously treated patients with AML was 24%, if all patients with this type of leukemia are considered. If early deaths are excluded, the CR rate was 27%. If patients with PRs are added and early deaths excluded, the CR plus PR rate is 32%. If the two patients with erythroleukemia are excluded, the patients with acute myeloblastic, myelomonoblastic, and monoblastic leukemias had a CR plus PR rate of 30%. Response rates to treatment with the combination of doxorubicin and ifosfamide are comparable to those seen with therapy with doxorubicin alone (2), where the CR plus PR rate for patients with previously treated acute myeloblastic, monoblastic, and myelomonoblastic leukemia was 31% (17 of 55 patients). Hence, the combination offers no demonstrable improvement in response rates over that achieved by the use of doxorubicin alone in AML variants.

The CR rate for previously treated patients with ALL was 89% (eight of nine patients) with the combination of doxorubicin and ifosfamide. Response did not differ whether the cell type was lymphoid or myeloid when doxorubicin was used as a single agent in previously treated patients with acute leukemia. The results of our study suggest that the combination of doxorubicin and ifosfamide may be particulary effective in ALL, and further investigations into the efficacy of the combination in ALL are warranted.

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Possible Relevance of *N*-Trifluoroacetyladriamycin (AD 41) in the Antitumoral Activity of *N*-Trifluoroacetyladriamycin-14-valerate (AD 32) in Tumor-Bearing Mice. I. Pharmacokinetic Evidence[1,2]

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SUMMARY

***N*-Trifluoroacetyladriamycin-14-valerate (AD 32) is an analog of doxorubicin whose chemico-physical characteristics are nontypical compared to the parent compound. Its most interesting feature is the lack of capacity to intercalate with DNA; thus, its mechanism of action as an antitumoral drug is still unknown. The *N*-trifluoroacetyl bond on the glycoside moiety is very stable and does not easily undergo enzymatic hydrolysis. Conversely, the valerate ester is split very rapidly by tissue and blood hydrolases. In this paper we present a kinetic study on AD 32, and we additionally follow the formation and disappearance of its metabolite, *N*-trifluoroacetyladriamycin (AD 41). Peak levels, areas under the curve, and β-half-lives of AD 32 and AD 41 after an iv injection of 80 mg/kg of AD 32 to Lewis lung carcinoma-bearing mice are presented. The results indicated very rapid disappearance of AD 32 from blood and tissues, whereas AD 41 persisted for much longer. Moreover, all of the tissues taken into consideration were able to hydrolyze AD 32 to AD 41, suggesting that this compound plays an important role in the antitumoral activity of AD 32.**

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The mechanism of action of *N*-trifluoroacetyladriamycin-14-valerate (AD 32) (fig 1), a relatively recent derivative of doxorubicin (Adriamycin), is still unclear. The original idea of Parker et al (1) and Israel et al (2) was to prepare a prodrug which would release doxorubicin slowly in the body and which might, at least in part, overcome the well-known cardiac toxicity of this drug. The result was AD 32, and though it has very important antitumoral activity (in some cases superior to the parent compound [3–5]), together with lower toxicity, it forms only traces of doxorubicin in vivo (6). Moreover, AD 32 possesses chemico-physical characteristics completely different from those of doxorubicin. The main effect of these differences in biologic systems is that AD 32 does not intercalate with DNA and is concentrated in cytoplasm (7–10).

The inability to intercalate DNA is linked to the trifluoroacetate amide present on the glycosidic moiety, since *N*-trifluoroacetyladriamycin (AD 41), ie, the same compound without the ester bond on position 14, does not intercalate to DNA either. The stability to enzymatic hydrolysis of the amide bond on the glycosidic moiety was demonstrated by Israel et al (6) who found not only AD 41 in bile and urine but also *N*-trifluoroacetyl-adriamycinol (AD 92) with this bond.

It has been demonstrated that AD 32 is rapidly biotransformed in the body and that, for instance, the half-life of this compound in mouse blood after a single iv injection of 80 mg/kg is 16 minutes, as

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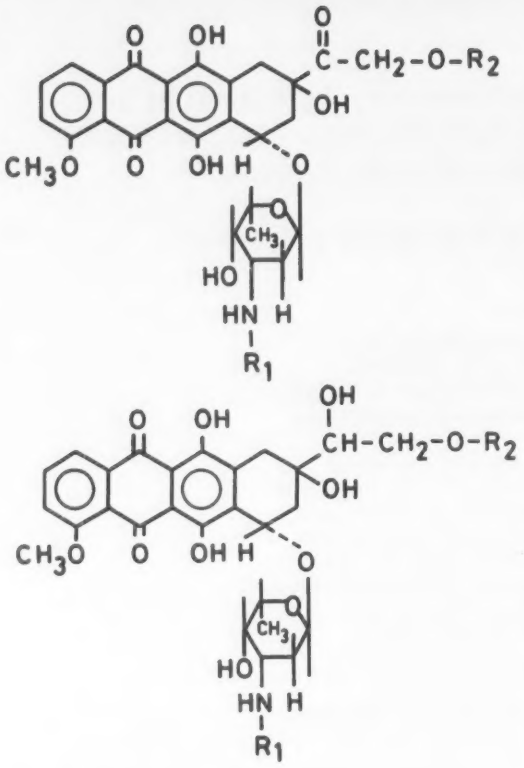

FIGURE 1.—Structural relationship between doxorubicin, AD 32, and metabolites. Top: doxorubicin, $R_1 = R_2 = H$; AD 32, $R_1 = COCF_3$, $R_2 = COCH_2CH_2CH_2CH_3$; AD 41, $R_1 = COCF_3$, $R_2 = H$. Bottom: adriamycinol, $R_1 = R_2 = H$; AD 60, $R_1 = COCF_3$, $R_2 = COCH_2CH_2\text{-}CH_2CH_3$; AD 92, $R_1 = COCF_3$, $R_2 = H$.

compared to 35 minutes for the main metabolite, AD 41 (11).

On the basis of these considerations it can be concluded that the first step of AD 32 biotransformation is due to nonspecific hydrolytic enzymes forming AD 41, and this compound is then metabolized to AD 60 and AD 92 but to a much lesser extent. The role played by AD 41 may be crucial with regard to the antitumoral activity since this is the substance which, in fact, is present in the organism for a relatively long time.

MATERIALS AND METHODS

Chemicals.—All solvents were reagent grade (Carlo Erba, Milan, Italy). Silica gel plates (20 × 20 cm, 0.25 mm thick) without fluorescence indicator and without calcium sulfate binder were purchased from E. M. Merck, Darmstadt, West Germany. AD 32, AD 41, doxorubicin, adriamycinol, and daunomycinone were kindly supplied by Farmitalia, Milan, Italy. AD 32 and AD 41 were dissolved at a concentration of 6 mg/ml in saline containing 10% Tween 80. AD 92 was a gift from Dr. Mervyn Israel, Sidney Farber Cancer Institute, Boston, MA.

Animals.—C57BL/6 male mice (body weight, 20 ± 2 g) obtained from Charles River Italy, Calco, Italy, were used for these experiments. The animals received an im transplant of 2×10^5 viable cells of the Lewis lung carcinoma, maintained by passages in the same strain every 2 weeks. AD 32 was administered iv at a dose of 80 mg/kg 14 days after tumor transplantation. Animals were killed at various intervals after drug administration (1, 3, 5, 10, 15, 30, and 60 mins and 2, 4, 5, 6, 12, 24, and 48 hrs) and blood, heart, tumor, and spleen were immediately collected. Tissues were repeatedly washed in chilled saline solution in order to eliminate blood contamination. At each time four animals were used.

Apparent V_{max} and K_m values.—Enzymatic hydrolytic parameters were determined as follows: tissues were homogenized at a ratio of 1:4 or 1:8 with 0.05 M phosphate buffer, pH 7.4, to obtain a final protein concentration of about 10–20 mg/ml. For incubation either 0.5 ml of heparinized blood or 0.5 ml of the tissue homogenate was added to 3 ml of 0.05 M phosphate buffer, pH 7.4, and preincubated for 5 mins at 37°C. The reaction was started by adding 0.5 ml of AD 32 dissolved in ethanol or in saline containing 10% Tween 80 to obtain a final substrate concentration ranging from 0.005 to 0.250 mM. After 5 mins of incubation at 37°C the reaction was stopped by adding 0.2 ml of cold acetone. AD 32 and AD 41 were extracted, dried, and quantitated as previously described (11). Enzyme activity was expressed as μmols of AD 41 formed per minute per milligram of protein. K_m and V_{max} values were determined according to the Woolf plot. Protein was measured according to Lowry et al (12).

AD 32 and AD 41 Assay

AD 32 and AD 41 were measured in blood and tissues by a specific, quantitative thin-layer chromatographic method, which has been described in detail elsewhere (11). These are the main features of this method briefly described. Blood was collected in heparinized tubes and immediately cooled to 0°C; 0.5 ml of blood was then mixed with 0.8 ml

of chilled water and deproteinized by the addition of 0.2 ml of cold acetone. The sample was extracted twice with 3 ml of ethyl ether, and the ethereal phases were pooled and dried under a gentle stream of nitrogen. Tissues were homogenized with an Ultra-Turrax apparatus, model TP 18/2N (Jane and Kungel, Stafen, W Germany) in chilled water at a ratio of 1:4 or 1:10 (g/ml), and 0.2 ml of cold acetone was added to 0.5–1.0-ml samples. The samples were extracted twice with 3 ml of ethyl ether and dried as described above. The residues were dissolved in 100–200 μl of an acetone solution of daunomycinone (20 μg/ml) used as internal standard and 5 μl of the solution was spotted on a silica gel plate using a micropipette. The silica gel plates (Merck) were developed to 15 cm from the origin in a lined tank and were then removed and dried briefly before scanning. The spots were quantitatively evaluated by means of a Turner III fluorescence scanner set at an excitation wave length of 475 nm and emission at 580 nm, connected with a Kontron W+ W 1001 recorder.

RESULTS

Figures 2 and 3 represent the kinetics and tissue distribution of AD 32 and AD 41 after a single iv injection of 80 mg/kg of AD 32 to Lewis lung carcinoma-bearing mice. Figure 2 shows the kinetics for blood, tumor, heart, and spleen, and figure 3 shows the kinetics for intestine, kidneys, lungs, brain, and liver. The curves report the levels of AD 32 and AD 41 until they were above the sensitivity of our method (0.150 μg/ml for blood and 1 μg/g for tissues). Table 1 shows the peak levels for both compounds, with the time at which they were measured, the areas under the concentration-versus-time curves (AUC) (which give a measure of the total amount of drug to which blood or tissues are exposed), and the β-half-lives. AD 32 and AD 41 behave in completely different ways. The general trend indicates rapid disappearance of AD 32 from blood and all other tissues taken into consideration. The peak levels indicate that AD 41 reaches higher levels than AD 32 in all tissues except blood, heart,

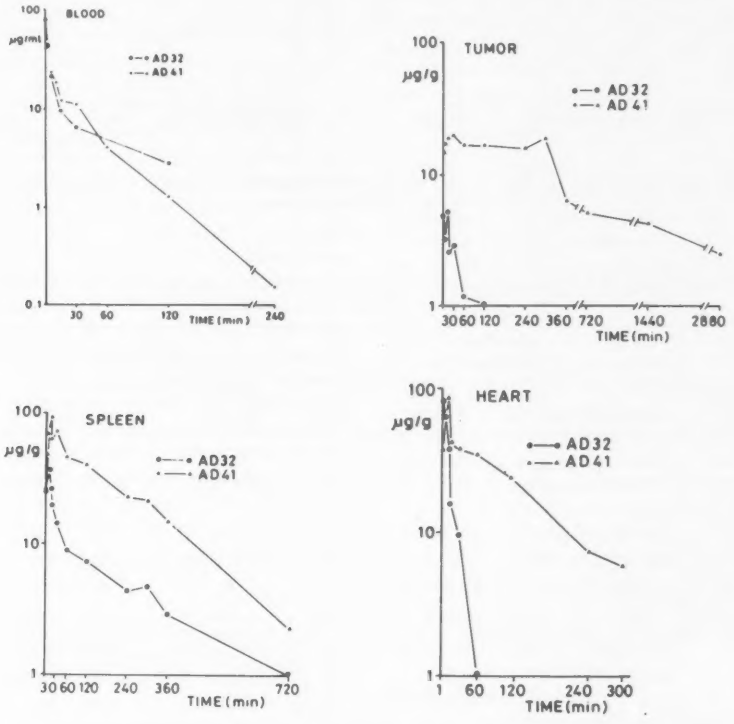

FIGURE 2.—Kinetics of AD 32 and AD 41 after a single iv injection of 80 mg/kg of AD 32 in blood, tumor, heart, and spleen in tumor-bearing mice.

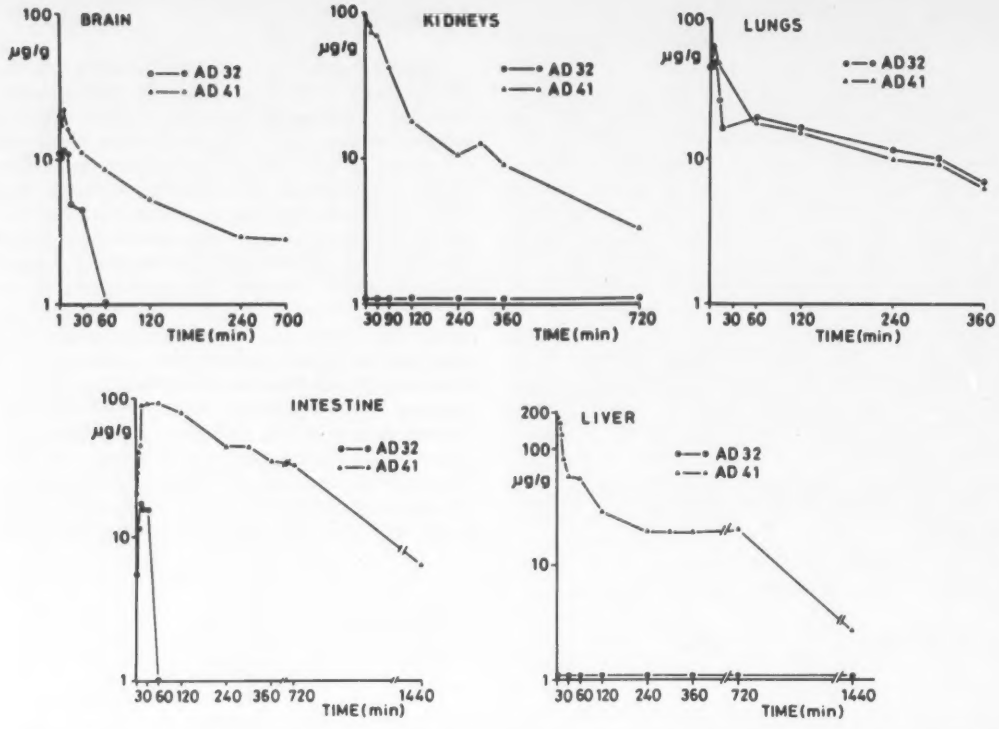

FIGURE 3.—Kinetics of AD 32 and AD 41, after a single iv injection of 80 mg/kg of AD 32 in intestine, kidneys, lungs, brain, and liver in tumor-bearing mice.

TABLE 1.—Blood and tissue levels of AD 32 and AD 41 after injection of AD 32 at a dose of 80 mg/kg iv to C57BL/6 mice bearing im Lewis lung carcinoma (treatment was given on Day 14 after transplantation)

Tissue	Peak level (µg/ml or g ± SE)				AUC* after 48 hrs (µg/ml or g × mins ± SE)		β-half-life† (hrs)	
	AD 32	Time (mins)	AD 41	Time (mins)	AD 32	AD 41	AD 32	AD 41
Blood	77.1 ± 2.4	1	44.8 ± 0.5	3	670.5 ± 65.5	990.45 ± 187.8	0.26	0.58
Tumor (Lewis lung carcinoma)	5.2 ± 0.6	10	19.3 ± 1.5	30	161 ± 15	16,078 ± 4197	0.34	5.82
Heart	89.3 ± 7.3	1	88.6 ± 2.8	10	947 ± 16	7136 ± 730	0.32	1.40
Spleen	36.5 ± 2.3	5	94.9 ± 11.8	10	2649 ± 113	15,920 ± 425	1.78	1.15
Liver	ND‡		193.2 ± 15.1	1	ND	27,896 ± 3781	ND	1.55
Kidney	ND		100.5 ± 4.8	3	ND	11,022 ± 1300	ND	4.08
Lung	84.9 ± 8.7	1	59.4 ± 2.3	5	5067 ± 580	5837 ± 139	3.29	2.20
Intestine	16.8 ± 1.6	10	83.3 ± 4.5	60	410 ± 63	46,652 ± 10,676	0.05	1.50
Brain	11.4 ± 1.7	5	22.3 ± 1.8	5	211 ± 30	1723 ± 20	1.62	1.51

*AUC was measured by trapezoidal integration up to the last time at which the drug was detectable.
†Half-life was found by calculating the significance of the regression using the determination coefficient r^2 (least-squares method).
‡ND = not detectable.

TABLE 2.—Apparent K_m and V_{max} values* of AD 32 hydrolysis by different tissues

Tissue	K_m (μM)	V_{max} (nmol/min/mg of protein)
Blood	9.78 ± 0.75	0.31 ± 0.002
Tumor	18.59 ± 1.91	0.41 ± 0.06
Heart	47.4 ± 4.92	0.21 ± 0.007
Spleen	10.12 ± 0.97	0.37 ± 0.003
Liver	17.15 ± 1.68	0.91 ± 0.011
Kidney	12.23 ± 1.52	0.58 ± 0.006
Lung	10.34 ± 1.04	0.60 ± 0.006
Intestine	18.34 ± 2.84	1.42 ± 0.038
Brain	9.14 ± 2.09	0.133 ± 0.002

*Each value = mean ± SE of 4 determinations.

and lung. In liver and kidney AD 32 could not be measured at all, indicating that hydrolysis in these tissues proceeds at a very high rate. Peak levels are reached by both drugs at quite different times: in general, AD 32 has a shorter half-life than AD 41, except in the brain. It is noteworthy that both AD 32 and AD 41 are found in the brain; the AD 41 peaks obtained are comparable to those in the tumor. The AUCs measured after 48 hours indicate that drug exposure of all tissues except lung is much higher for AD 41 than for AD 32. The difference between the AUCs is important and amounts to a whole order of magnitude except in blood and lungs.

The β-half-life confirms the tendency already described and indicates that AD 41 persists longer in all tissues except spleen, lung, and brain.

Table 2 reports the apparent K_m and V_{max} values for the hydrolysis of AD 32 to AD 41. The blood and brain have the lowest apparent K_m values, 9.14 and 8.78 μM, respectively. The heart shows the highest apparent K_m value, 47.4 μM. All other tissues have intermediate apparent K_m values ranging from 10.12 to 18.59 μM. Strangely enough, the intestine has the highest V_{max} value, 1.42 nmols/min/mg of protein, much higher than the 0.91 nmol/min/mg of protein in liver. The brain has the lowest value, 0.13 nmol/min/mg of protein, and the values for the other tissues range from 0.21 to 0.60 nmol/min/mg of protein.

DISCUSSION

The aim of this study was to quantitatively assess the importance of AD 41 in the antitumoral action of AD 32. Although pharmacokinetic studies cannot give a conclusive answer to this question, the results reported in this paper tend to stress the importance of AD 41.

The therapeutic role of AD 32 as such or as a prodrug of doxorubicin is presumably very small because it is rapidly hydrolyzed to AD 41 by hydrolases present in tissues, and because no doxorubicin was ever found in our study after treatment with AD 32. This is in good agreement with the metabolic studies performed by Israel et al (6,13), Pegg et al (14), and Wilkinson et al (15) who showed that, after AD 32 treatment of rats and mice, doxorubicin was found only in traces in urine and bile; no pharmacologic importance can be ascribed to this minimal quantity. Conversely, AD 92 and AD 60, two minor metabolites of AD 32, are found only in liver, urine, and bile, but so far there are no reports of their presence in other tissues, especially tumor. During our studies we noticed a peak in blood and tissues probably corresponding to AD 92. We were unable to perform quantitative determinations because of the lack of pure standard.

An interesting feature of both AD 32 and AD 41 is that they cross the blood-brain barrier. Since brain has very low hydrolytic specific activity, the amount of AD 41 found is probably partly due to hydrolysis in loco and partly due to the contribution of blood esterases. In this connection, as reported in our previous paper, we were obliged to measure AD 32 and AD 41 in whole blood because otherwise all of the remaining AD 32 is rapidly hydrolyzed during plasma preparation.

The slight difference of polarity between AD 32 and AD 41 may be important in determining their different distribution and accumulation in tissues. Although the antitumoral activity of AD 41 has been studied only to a very limited extent, there is some evidence that it has different pharmacologic activity and greater secondary effects (16). Studies on the comparative in vitro cytotoxicity of AD 32 and AD 41 on cultured Lewis lung carcinoma cells indicated that doses of AD 41 ranging between 0.5 and 50 μg/ml had a much greater effect, judging by cell-kill and inhibition of thymidine incorporation after treatment. If this trend were confirmed, it could be speculated that AD 32 is simply a carrier which accumulates rapidly in tissues, particularly in tumor, and is immediately hydrolyzed.

The contribution of blood hydrolases in the hydrolysis of AD 32 is difficult to evaluate. We are, at present, studying the kinetics of AD 41 administered as such with the aim of obtaining an answer to this question and determining whether tissue distribution is, in fact, different.

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Metabolism and Disposition Studies of 9-Hydroxyellipticine and 2-Methyl-9-Hydroxyellipticinium Acetate in Animals[1,2]

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SUMMARY

Radiolabeled 9-hydroxyellipticine (iv and ip routes) and the corresponding radioactive quaternary salt, 2-methyl-9-hydroxyellipticinium acetate (iv route), were administered to mice. Tissue distribution was then followed up to 64 hours by means of autoradiography. Both drugs accumulated in the kidneys, lungs, and liver; 9-hydroxyellipticine also accumulated in the spleen and bone marrow. The quaternary salt was concentrated in the gastrointestinal walls and the salivary and thyroid glands; none was found in the brain. Metabolic studies after administration of these antitumor drugs to rats and mice showed that 9-hydroxyellipticine is extensively metabolized, mainly to its glucuronide. Under our experimental conditions, the ellipticinium derivative was excreted unchanged in the bile (70%) and in the urine (30%). Pharmacokinetic studies in mice using either radioactivity measurements or selective extraction followed by spectrofluorometric quantitation have shown that blood levels drop very rapidly during the distribution phase followed by a much slower disposition phase, with a half-life of about 30 hours for the quaternary salt.

[Cancer Treat Rep 64:879–887, 1980]

Ellipticine and several of its derivatives have shown anticancer properties in various experimental tumor systems (1,2). Among these derivatives, 9-hydroxyellipticine (9-OHE) (fig 1) demonstrated antitumor activity in animals (3,4) but was found inactive in man (5). This hydroxy derivative was first prepared from 9-methoxyellipticine (4) on the basis of a rational design (3). Later, it was identified as a metabolite of ellipticine itself (6–8). However, another derivative, *N*-2-methyl-9-hydroxyellipticinium acetate (NMHE) (fig 1), has yielded some favorable results in preliminary clinical trials (9), mainly on osseous metastases of breast cancer. This compound has now entered phase II clinical trials under the supervision of the European Organization for Research on Treatment of Cancer, which reported the preliminary phase I results (10).

The difference in the in vivo activities of 9-OHE and NMHE in animals and in man (4) might result partly from their respective fates in the body and from their routes of administration. Therefore, we have undertaken preliminary pharmacokinetic studies of these two derivatives in animals. Their metabolism and tissue distribution will be compared to the results obtained previously with ellipticine (8,11).

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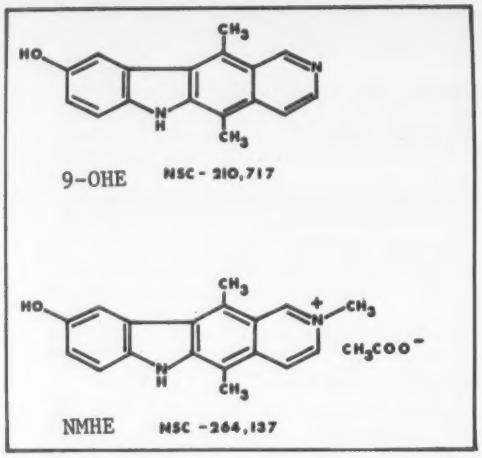


FIGURE 1.—Chemical structures of 9-OHE and NMHE.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]-9-OHE was synthesized by Van-Bac et al (12) according to the method of Dalton et al (1) modified by Dat-Xuong et al (13). [2-*N*-^{14}C]-NMHE was prepared through alkylation of 9-OHE with [^{14}C]-methyl iodide according to Le Pecq et al (14). Specific activity was 2.0 and 2.2 millicuries/mmol, respectively. β-Glucuronidase (arylsulfatase-free) and β-glucuronidase with arylsulfatase were purchased from Boehringer (Paris, France). Solvents and reagents used were of the best available quality (Baker Chemicals, Deventer, Holland or Carlo-Erba, Italy). Thin-layer chromatography (TLC) plates coated with Kieselgel 60 (Merck, Darmstadt, W Germany) were used to check the purity (> 99%) of the labeled compounds after elution with a mixture of *n*-butanol:water:acetic acid (4:4:1, vol/vol, upper phase).

Animals

Female DBA/2 mice (Centre National d'Elevage des Animaux de Laboratoire, CNRS, Orléans, France), weighing 19–20 g, were administered [1-^{14}C]-9-OHE dissolved in saline or [2-*N*-^{14}C]-NMHE dissolved in 0.1 M acetate buffer (pH 5.0); 9-OHE was injected either iv (caudal tail vein) or ip (peritoneum) at a dose of 25 mg/kg; the LD for mice is 50 mg/kg ip and 70 mg/kg iv (15). NMHE was administered by the iv route only at a dose of 15.5 mg/kg; the LD for DBA/2 mice is 5 mg/kg iv (14) and 7 mg/kg for Swiss mice (unpublished results). This dose was chosen because the specific activity was quite low.

The animals were housed in stainless steel metabolism cages to collect urine and feces. Blood samples were obtained in heparinized tubes by decapitation.

For biliary excretion studies of 9-OHE and NMHE, the two compounds were injected iv into 3-month-old male Wistar rats, weighing approximately 200 g, which were catheterized with a biliary cannula after anesthesia with urethane. They were given the same dose as mice, on a body-weight basis.

Autoradiography

Autoradiographic slices were obtained according to the original procedure of Ullberg (16). At different times after injection of the labeled compounds, the mice were dipped into an alcohol and dry ice bath (–65°C) after anesthesia with diethyl ether. Each animal was then sliced (cryotome Leitz 1300 K, Wild-Leitz, Rueil Malmaison, France) in the cold, into 40-μm thick preparations. The slices were then carefully layered on cellulose acetate strips (Scotch Minnesota Mining and Manufacturing Co, Paris, France) and dried for 5–6 days at –20°C. Radiologic films (Kodak Kodirex, 18 × 24 cm, Kodak-Pathé, Vincennes, France) were then tightly applied to these preparations and left in the dark at a cold temperature. Developing was done 13–15 days later. Two mice were used for each time point.

Extraction From Biologic Fluids and Tissues and Quantitation of Drug Levels

9-OHE.—9-OHE was extracted twice from whole-blood samples (1 ml) with ethyl acetate (2 ml) after treatment with 4 ml of 0.1 M borate buffer (pH 9.2). After shaking for 1 minute on a Vortex mixer (Scientific Industries, Inc, Bohemia, NY) and centrifugation for 10 minutes at 3000 rpm, an aliquot of the combined organic phases was analyzed in a spectrofluorometer using a photon-counting device built in the Laboratoire de Pharmacologie Moléculaire, Institut Gustave-Roussy. The excitation and emission wavelengths were 410 and 470 nm, respectively. The same procedure was used for homogenized tissues after dilution with 1 vol of borate buffer and subsequent extraction was carried out using 2 vol of ethyl acetate. After centrifugation

(3 minutes at 4000 × *g*), the supernatant was collected. The lower phase was subsequently washed with an additional volume of solvent and the organic layers were combined. This procedure is used to quantitate the unchanged drug only. The limit of sensitivity of the assay depends on the blank for each tissue. But the lowest concentration that can be accurately determined is 25 ng of 9-OHE/ml of blood with a standard deviation of 12% for triplicate determinations and 50 ng of 9-OHE/g of tissue. In addition, no metabolite can be extracted by this procedure. Recovery from blood is quantitative after two subsequent extractions with ethyl acetate, as shown in control experiments using the corresponding radiolabeled compounds; from tissue homogenates, the mean recovery is 85%–90%. This method is a modification of the technique described by Hardesty et al (17) for ellipticine itself.

NMHE.—NMHE was selectively extracted from total blood and tissues by means of an ion pair, using 0.05 M sodium tetraphenylborate and ethyl acetate. The organic layer was dried under nitrogen stream and the residue was treated with acetic anhydride in pyridine. The excess reagent and pyridine were evaporated in vacuo and the acetoxy derivative was quantified by spectrofluorometry (excitation and emission wavelengths at 452 and 540 nm, respectively) (18). Control experiments with radiolabeled NMHE proved the extraction procedure from blood to be quantitative under these conditions; about 90% is recovered from tissue homogenates.

Total Radioactivity Measurements in Tissues

The different organs of mice were deep-frozen in dry ice, then reduced to powder in a grinder and kept at –20°C until the time of analysis. A weighed aliquot of each tissue was burnt in an Oxymat apparatus (Intertechnique, Plaisir, France) and the radioactive CO_2 was trapped in 20 ml of a mixture of phenylethylamine (330 ml), toluene (400 ml), methanol (220 ml), water (50 ml), and butyl PBD (7 g) (Packard Instruments Co, Inc, Downers Grove, IL). The amount of radioactivity was then measured in a scintillation counter (Intertechnique). Each determination represents the average of two assays, with a pool of two tissues per assay. The third mouse of each group was used for measurement of the residual radioactivity.

Metabolic Studies

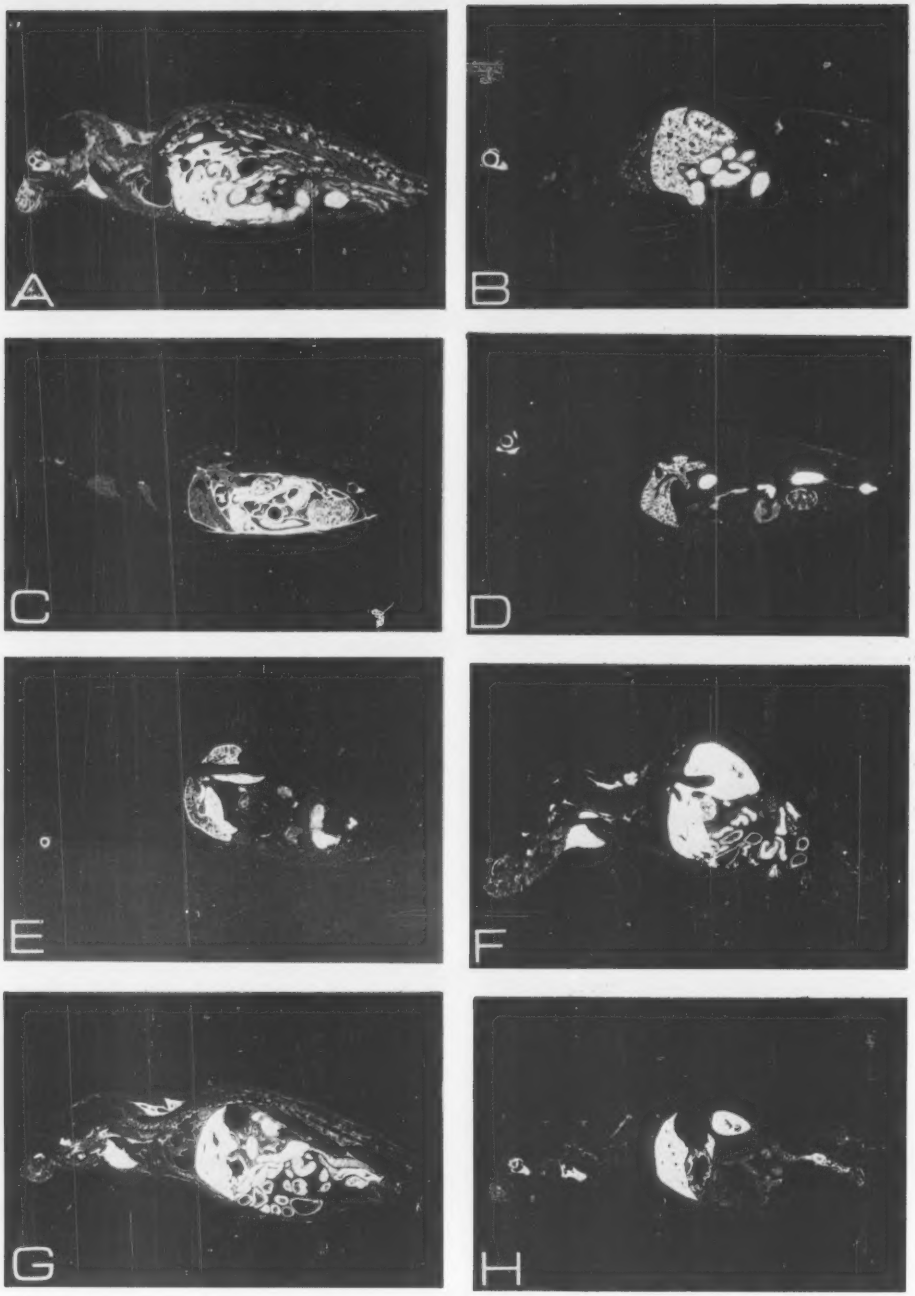
The elimination of 9-OHE and its metabolites through the biliary route was studied in catheterized rats. The bile from three rats was collected for 4 hours and pooled (a total of 10 ml). The lipids were extracted with chloroform and it was verified that no radioactivity had been extracted into this solvent. Some traces (0.3% of the administered dose) were removed with ethyl acetate after alkalinization (pH 9.0) and corresponded to unchanged drug. The residue was then purified (46% of the dose) on a column with cationotropic alumina (Woelm Pharma, GmbH, Eschwege, W Germany), the column being first percolated with 0.1 M acetic acid to discard the biliary salts, then washed with water. Finally, the pool of metabolites was eluted with 0.1 M sodium hydroxide. It was then diluted four times in a 0.2 M acetate buffer (pH 4.6) and incubated for 2 hours at 25°C with β-glucuronidase. The resulting mixture was then brought to pH 9.0 by addition of 0.1 M borate buffer. Extraction with ethyl acetate yielded a compound which was identified as 9-OHE by comparison with an authentic sample (TLC and mass spectrometry). In parallel experiments, the metabolites were hydrolyzed with β-glucuronidase with arysulfatase at pH 4.5 for 2 hours at 25°C, then for 2 additional hours at 38°C. The resulting mixture was treated as above.

RESULTS

Autoradiographic Studies

The tissue distribution of these two compounds was qualitatively compared by autoradiographic studies after iv and ip injections of 9-OHE and iv administration of NMHE. Results are shown in figure 2A–L. After iv administration, both drugs accumulated in the kidneys, lungs, and liver, but 9-OHE accumulated largely in the spleen, bone marrow, around the ocular globe, and inside the intestinal lumen (fig 2A, B, and D). Furthermore, it appeared that body distribution of 9-OHE depended on the route of administration. When given ip, the drug remained sequestered inside the peritoneal cavity, which may act as a deep compartment (fig 2C), thus delaying the distribution to other tissues. With the iv route, at the same time (ie, 5 hours after administration), we note that the compound was concentrated mainly in the lungs, liver, and kidneys (fig 2B). At later times (16 and 48 hours), traces of 9-OHE radioactivity were detected in the intestine contents and in the liver (fig 2D and E) whatever the route of administration.

The quaternary compound, NMHE, rapidly distributed to the walls of the stomach and the intestines (fig 2F), as well as to the thyroid and salivary glands. In two different mice (fig 2I and J), 16 hours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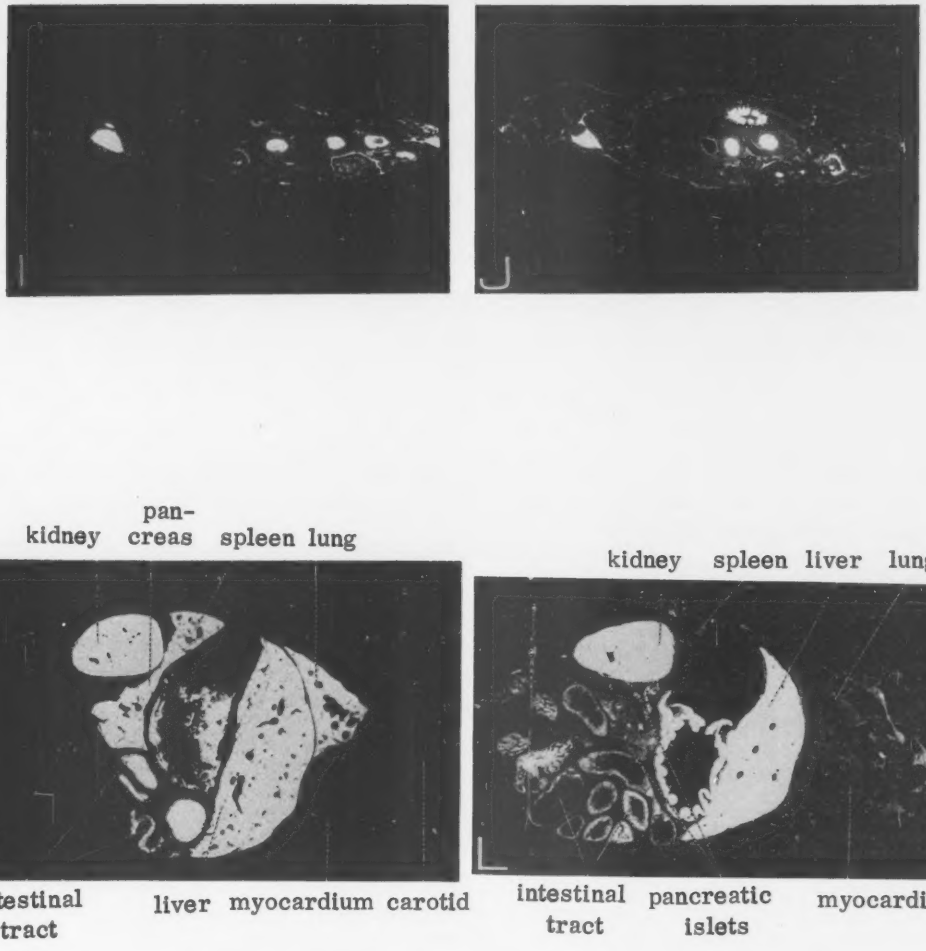

FIGURE 2A–L.—Whole-body autoradiograms obtained in mice: A) 30 mins after iv administration of 9-OHE; B) 5 hrs after iv administration of 9-OHE; C) 5 hrs after ip administration of 9-OHE; D) 16 hrs after iv administration of 9-OHE; E) 48 hrs after ip administration of 9-OHE; F) 5 mins after iv administration of NMHE; G) 30 mins after iv administration of NMHE; H) 30 mins after iv administration of NMHE; I) 16 hrs after iv administration of NMHE; J) 16 hrs after iv administration of NMHE; K) details obtained 30 mins after iv injection of 9-OHE; L) details obtained 30 mins after iv injection of NMHE.

after injection of NMHE, radioactivity is mostly concentrated in the intestinal lumen and in the kidneys.

In a control experiment, oral administration of a radiolabeled 9-OHE solution to mice by means of gastric tubing indicated that oral bioavailability is nil since no radioactivity was found outside the gastrointestinal tract at 2, 4, and 8 hours after administration (results not shown).

Metabolic Studies

9-OHE.—In three rats, after collection of the bile (10 ml during the first 4 hours) and following enzyme hydrolysis of the metabolites, the major metabolite formed after administration of the drug was the glucuronide (90%–95% of the amount excreted in the bile) corresponding to 46% of the dose. We have not found any significant difference in radioactivity measurements after treatment with β-glucuronidase alone or β-glucuronidase with arylsulfatase. However, we cannot exclude the possible presence of a small amount (5%) of the sulfate because of experimental error (±5%). In the feces of noncatheterized rats, only unchanged 9-OHE could be recovered, which means that the glucuronide has been hydrolyzed in the intestines. In three mice, most of the radioactivity was also excreted unchanged in the feces and the amount collected in urine accounted for only 8%–12% of the dose over 64 hours (mainly as the glucuronide). Analysis of three blood samples from a rabbit, either by total radioactivity (dosage of unchanged drug plus metabolites) or by selective extraction of the unchanged 9-OHE (spectrofluorometric assay) shows that there was a significant difference between these two analytic methods, indicating that the glucuronide was circulating in the bloodstream.

NMHE.—Similar experiments conducted in three rats and three mice with NMHE proved that this compound was not metabolized to any detectable extent and was excreted as the parent compound in the bile (70% of the excreted amount) and in the urine (30%). Within 64 hours, 85% ± 8% of the dose had been eliminated.

Pharmacokinetic Studies

9-OHE.—After iv administration, total radioactivity or selective extraction and spectrofluorometry were used to monitor the tissue levels as a function of time. Results (total radioactivity) are shown in figure 3 for the main organs. It shows that after a rapid distribution phase, a much slower disposition phase follows. However, care must be taken in interpreting these data on the basis of total radioactivity measurements, since these measurements correspond to the parent compound and its glucuronide (and possibly traces of sulfate). However, since this compound was not to be used in man any longer, we did not analyze every blood sample for unchanged drug. 9-OHE was eliminated in the bile (88%) and by the kidneys (12%) in 64 hours, during which time 90% of the dose had been eliminated. At 64 hours, the total radioactivity still present in mice (determined in whole animals) was 3.6% of the administered dose after ip injection and 1.7% after iv administration.

NMHE.—Total radioactivity, in this case, was used as a quantitative determination of the parent compound in mice since no metabolite could be detected under our experimental conditions. Figure 4 shows the concentration decay pattern (after iv injection) of this labeled compound in the main organs as a function of time. As with 9-OHE, these curves display a multiphasic pattern, the terminal half-life in blood being about 30–35 hours. The blood level-time plots are quite similar to those for other antitumor compounds such as the anthracyclines (doxorubicin, daunorubicin) (19,20). It must be noted that in some tissues (liver, lungs, and kidneys) concentrations are about ten times the concentration in blood, and correspond to in vitro levels that are cytotoxic in L1210 leukemic cells (21). After 64 hours, the radioactivity still present in the animals accounted for 13% of the administered dose. This agrees well with the calculation of the areas under the blood level-time plot (calculated: 17%) and with the fact that a total of 80% of the dose had been eliminated either in the feces (70%) or in the urine (30%).

DISCUSSION

Hardesty et al (17) studied the effect of the route of administration (oral and ip) on the tissue distribution of ellipticine in mice. They found very poor adsorption after oral administration, and little renal excretion (< 1% in 5 days).

Later, Chadwick et al (11) compared the physiologic disposition of ellipticine in several animal species after iv administration. All species excreted 80% of the dose via the fecal route and 10% of the dose via the urinary route, primarily as glucuronide and sulfate.

When studying physiologic distribution of 9-OHE and NMHE in mice, we noted that this process was very fast when the drug was given as an iv bolus. However, after ip administration of 9-OHE, distribution was delayed due to its intraperitoneal se-

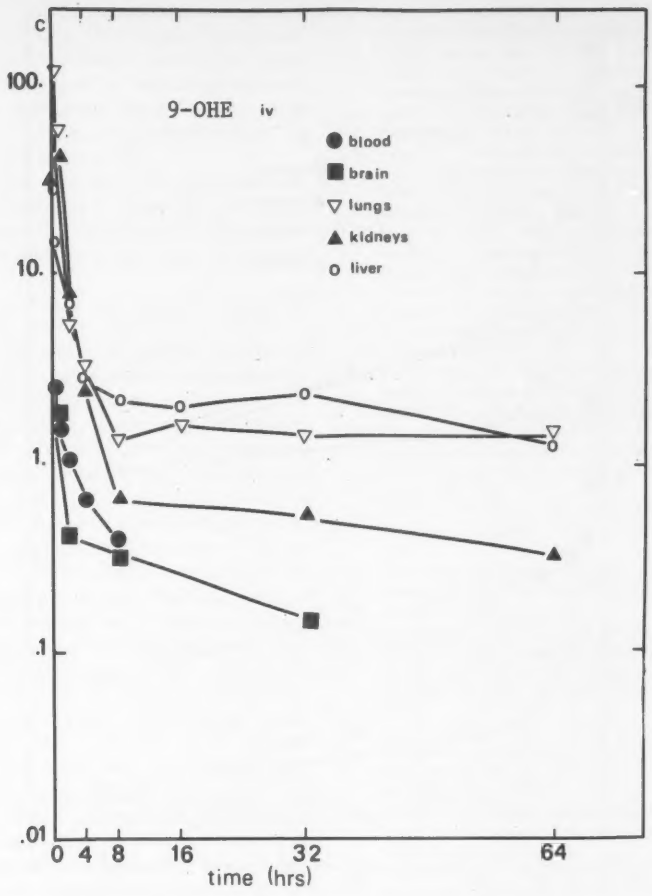

FIGURE 3.—Semilogarithmic tissue concentration-time plots after iv injection of 9-OHE into mice. Values on the ordinate = μg equivalents/ml of blood or g of tissue.

questration (fig 2C). This phenomenon might explain the previously observed differences in the toxicity and the efficacy on animal tumors (4,18) depending on the route of administration. The sublethal ip dose (50 mg/kg) is different from the sublethal iv dose (70 mg/kg) (15). We have also shown (4) that L1210 cells located in the CNS can better be reached if the drug is injected iv.

Furthermore, long-lasting concentrations of 9-OHE were found around the ocular globe (fig 2A–E), regardless of route of administration. This phenomenon deserves more precise histologic studies but can be related to epidermis fixation since both tissues contain melanocytes.

Elimination of 9-OHE as its glucuronide via the biliary route indicates that the liver is the primary site of biotransformation of this compound. However, when the metabolite is secreted into the intestinal lumen, it undergoes enzyme hydrolysis since, instead of metabolite, intact drug was found in the feces of noncatheterized animals. The slow elimination phase (fig 3) is unlikely to be due to enterohepatic re-circulation because studies of orally administered 9-OHE (unpublished) have proved that it is very poorly absorbed through the intestinal mucosa as ellipticine (17); the slow elimination phase would rather be due to tissue binding. Analysis of tissue contents, either by total radioactivity

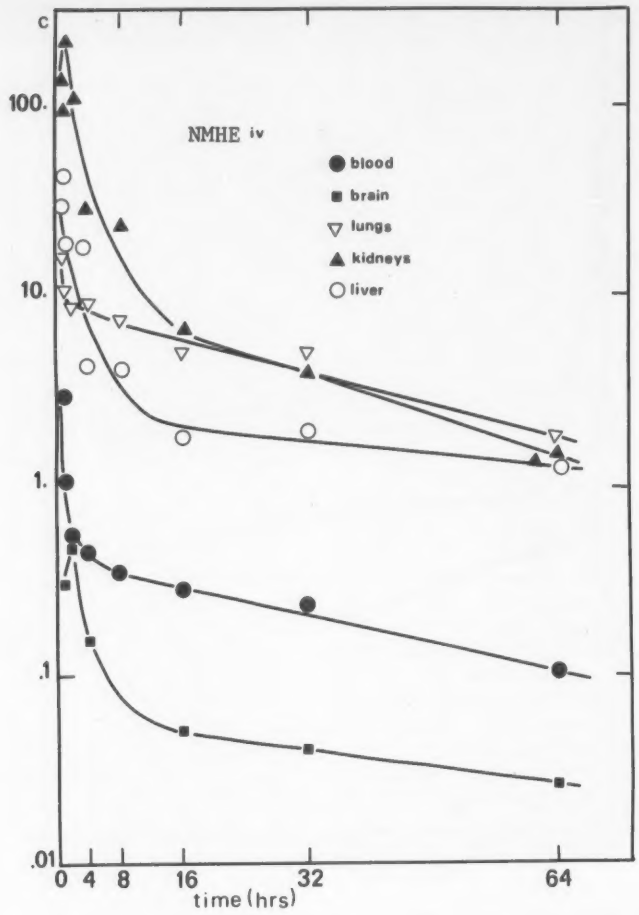

FIGURE 4.—Semilogarithmic tissue concentration-time plots after iv administration of NMHE to mice. Values on the ordinate = μg equivalents/ml of blood or g of tissue.

measurements or using our specific spectrofluorometric assay indicates that the glucuronide is also widely distributed.

The amount excreted renally is quite low (8%–12% of the dose) after 64 hours; this amount corresponds mainly to the glucuronide.

Contrary to 9-OHE, the quaternary derivative, NMHE, was not metabolized to any detectable extent under our experimental conditions: neither glucuronide nor sulfate was found; no evidence of biotransformation of the quaternary group or reduction of the quaternary salt was recorded. However, this observation does not rule out the possibility of an oxidative generation of free-radical species from NMHE which would have a short half-life and revert back to the initial drug by reduction. Evidence in support of the in vitro generation of such free radicals from 9-OHE and NMHE has been obtained (C. Auclair, C. Paoletti: unpublished data).

Therefore, in our in vivo studies with NMHE, the total radioactivity levels are likely to represent the intact compound and are suitable for disposition studies. The terminal half-life determined in blood (nonlinear regression) is about 30–35 hours. This result led us to undertake pharmacokinetic studies in man (to be published) to check the risk of accu-

mulation arising from a continuous treatment schedule (80–100 mg/m² weekly), keeping in mind, however, that the doses used in animals (mice and rats) approached or exceeded the infralethal dose and may thus involve saturable processes regarding tissue distribution and disposition.

Despite the fact that NMHE is not metabolized, the main route of elimination is the feces (70% of the total excreted) and the renal excretion accounts for 30%, since 85% of the total dose was eliminated in 64 hours.

For NMHE, we noted a quick accumulation in the thyroid gland (fig 2F, H, and I) that may be of clinical significance (9) and a deposition in the salivary glands that may be responsible for mouth dryness, a side-effect common to most patients (10).

In addition to these differences, two other characteristics of 9-OHE versus NMHE have recently been reported: (a) NMHE does not bind to liver microsomal cytochrome P-450; 9-OHE does. (b) NMHE does not mutate the bacterial strains in the Ames assay in the presence or absence of microsomes (7,19), while 9-OHE is reactive. Whether the lack of NMHE reactivity versus the reactivity of 9-OHE in the microsomal system is related to some differences between these two compounds in metabolism pharmacokinetics or antitumor efficacy is still unknown. However, more detailed studies are underway in our laboratory to prepare larger quantities of pure 9-OHE glucuronide in order to test it against L1210 leukemic cells in culture and against some experimental tumors. Although glucuronides have long been considered inactive species, the potential antitumor or toxic activity of 9-OHE glucuronide should be checked. Indeed, it has been shown that some tissues or tumors (20,22,23) contain high levels of β-glucuronidase and likewise that the reaction intermediate (a radical) of theglucuronide hydrolysis may be highly reactive (24).

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Recovery From Potentially Lethal Damage Induced by Spirohydantoin Mustard on 9L Cells In Vitro[1,2,3]

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SUMMARY

Spirohydantoin mustard (SHM) has been shown to cross the blood-brain barrier in dogs and has shown activity against mouse brain tumors in vivo and against 9L rat brain tumor cells in vitro. Factors influencing the recovery of monolayer 9L cells from SHM-induced potentially lethal damage were studied. The pattern of recovery was similar in exponentially growing and plateau-phase cultures. Recovery occurred in either Earle's balanced salt solution (EBSS) or depleted medium (DM), and was inhibited in fresh medium (FM). The recovery process had a half-life of ~4 hours and was completed within 18 hours. The enhancement of survival was dependent on drug concentration and temperature. The magnitude of recovery decreased as the temperature was lowered and was inhibited at ambient temperature, which suggested that an enzymatic repair process was responsible for recovery. The distribution of cells through the cell cycle after SHM treatment was determined using a flow cytometer. Incubation of exponentially growing and plateau-phase cells in either EBSS or DM impeded the progression of cells from G_1 into S phase, but cells incubated in FM progressed into S phase by 24 hours. We conclude that the recovery occurred primarily when cells were in G_1 phase and potentially lethal damage would be fixed when cells initiate DNA synthesis within 18 hours after administration of SHM. Therefore, optimal therapeutic benefit may depend on drug scheduling.

[Cancer Treat Rep 64:889–895, 1980]

Spirohydantoin mustard (SHM), a drug in which 2-ethyl nitrogen mustard is linked to the 3-nitrogen of spirocyclohexyl hydantoin, has been shown to cross the blood-brain barrier of dogs (1) and to be effective against several murine tumors in vivo (including an intracerebral ependymoblastoma [2]) and against 9L rat brain tumor cells in vitro (3,4). However, single doses of SHM have not been effective to date in increasing the lifespan of rats bearing the 9L brain tumor (4).The survival curve of 9L cells treated with SHM is characterized by an initial shoulder followed by an exponential region of cell-kill and, at concentrations of > 9 μg/ml, by a plateau region at surviving fractions (SFs) of $\sim 10^{-4}$ (4). This

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probably indicates that a small fraction of the cell population is drug-resistant. Exponentially growing and plateau-phase cultures appear to be equally sensitive to the drug, and preliminary experiments indicated that this cell line can recover from SHM-induced potentially lethal damage (PLD) (4).

Recovery from PLD is measured by an increase in survival resulting when the survival assay is delayed for various "recovery" times after a single dose of the agent (5–9). Recovery from PLD in vitro has been observed in exponentially growing and plateau-phase cultures (8,10), in oxic and hypoxic cells (6), and in monolayer and spheroid culture systems (7). Usually, enhancement of survival occurs for various post-treatment conditions that are suboptimal for growth, such as depleted medium (7,8), balanced salt solution (6,11), low temperature (12,13), and inhibition of protein synthesis by cycloheximide (9). However, lowering the temperature has also inhibited the recovery phenomenon (9,11), and post-treatment exposure to cycloheximide has not always increased survival (11). Inhibition of recovery has been observed with various post-treatment conditions such as fresh medium (6–8), short exposure to high temperature (14), anisotonic solutions (15), and extreme hypoxia (16). Post-treatment exposure of cells to inhibitors of DNA synthesis (5-fluorodeoxyuridine, hydroxyurea) (9) or to inhibitors of DNA-directed RNA synthesis (actinomycin D) (17) inhibits the recovery process, although this observation has not been universal (11).

These conflicting results of the influence of temperature and metabolic inhibitors on the recovery process strongly suggest that the magnitude of recovery from PLD is as dependent on the cell line as on the treatment and post-treatment conditions. This study investigated the factors influencing the recovery from SHM-induced PLD of 9L cells in vitro.

MATERIALS AND METHODS

Cell Culture Conditions

The 9L stock cultures were maintained as monolayer cells in complete fresh medium (FM) consisting of Eagle's minimum essential medium supplemented with 10% fetal calf serum, 87 μg/ml of streptomycin, and 87 units/ml of penicillin in a 95% air-5% CO_2 humidified incubator at 37°C.

Exponentially growing cultures were obtained by seeding 2×10^6 cells into 75-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) containing FM, 24 hours before the drug treatment; there were ~3.5×10^6 cells/flask at the time of treatment. Plateau-phase cultures were obtained by seeding 1×10^6 exponentially growing cells into 75-cm^2 tissue culture flasks containing FM where they grew unfed until the experiments were performed 5 days later. The number of cells per flask increased exponentially during the first 3 days and then reached a plateau of ~1.6×10^7 cells/flask on Day 4, where it remained for at least 3 more days.

Survival Determination

Survival was determined using the colony-formation assay described previously for this cell line (4). After drug treatment or after the time allowed for recovery from PLD, cells were trypsinized with saline A containing 0.05% trypsin and 0.02% versene for 5–10 minutes, and a single-cell suspension in FM was prepared. The optimum number (5×10^4) of irradiated 9L feeder cells and the optimum incubation time (12–14 days) were determined previously (4).

Plating efficiency (PE) for treated and untreated cells was calculated as the ratio of the number of colonies counted to the number of cells plated. SFs were calculated as the ratio of the PE of treated cells to the PE of untreated cells.

Recovery From Potentially Lethal Damage

Following SHM exposure, one group of control and one group of treated cells were plated immediately for colony formation. Other control and treated groups were washed once with 15 ml of buffered Earle's balanced salt solution (EBSS) at 37°C and allowed to recover up to 28 hours in either FM, EBSS, or depleted medium (DM), which was collected from flasks of plateau-phase cultures and filtered to remove cells and debris. Except when noted, during the recovery period the cells were maintained in the humidified incubator at 37°C. In the temperature-effect studies, flasks were maintained at ambient temperature (~23°C) or immersed in a 30°C or 40°C Dubnoff metabolic shaking incubator, which was accurate to ±0.5°C (Precision Scientific, Chicago, IL). The pH was monitored and maintained at 7.2–7.3.

After various recovery times, cells were trypsinized and plated for colony formation. All experiments were repeated twice. Recovery factors (RFs) were calculated from the expression S_t/S_o, where S_t is the SF of cells plated after various recovery times and S_o is the SF for cells plated immediately after treatment.

Drug Treatment

SHM was dissolved in ethanol and diluted with FM. The final concentration of solvent in the treatment flasks was < 1%, which is not toxic to 9L cells. Cells were treated for 1 hour at 37°C and at pH 7.2–7.3.

Cell Progression Analysis

Samples from each flask used for PLD experiments (5 μg/ml of SHM) were harvested, fixed with 70% aqueous ethanol, and stained for a minimum of 20 minutes with mithramycin (Pfizer, Inc, New York, NY) as described previously (18). A flow cytometer (FACS III, Becton-Dickinson, Mt View, CA) was used to analyze DNA profiles of ~50,000 cells/sample at an excitation wavelength of 457 nm. The coefficient of variation (CV) for 2.0 μm latex microspheres (Duke Scientific, Palo Alto, CA) was 2.0%.

RESULTS

Effect of Drug Concentration

Exponentially growing cultures, treated with graded concentrations of SHM and held for 24 hours in EBSS at 37°C, had a higher SF than cells plated immediately after treatment (fig 1). The enhancement of survival, expressed as RF, increased exponentially with increasing concentrations of SHM to a maximum at 7 μg/ml (fig 2). However, the RF was less at 9 μg/ml than at 7 μg/ml. RFs observed in these experiments were lower than those previously reported (4), and probably reflect the greater cell-kill achieved with SHM in the earlier experiments.

Effect of Recovery Medium

A 2-log cell-kill resulted when either exponentially growing or plateau-phase cultures were treated for 1 hour at 37°C with 5 μg/ml of SHM and assayed immediately for survival. For both exponentially growing (fig 3) and plateau-phase (data not shown) cultures, recovery was inhibited when the cells were overlaid with FM during the recovery period, but occurred when the cells were overlaid with either EBSS or DM. The kinetics of recovery and the final survival level attained were similar for both types of cultures (RFs of 15–19). A complete time profile for exponentially growing cells in EBSS (fig 4) shows a recovery half-life of ~4 hours and a plateau (RF~19) for all times > 18 hours.

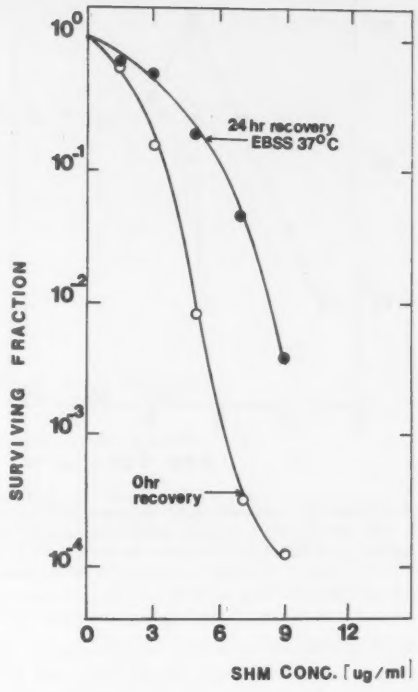

FIGURE 1.—Survival curve of exponentially growing 9L cells treated for 1 hr with graded concentrations of SHM and either trypsinized immediately after treatment (lower curve) or held in EBSS at 37°C for 24 hrs before trypsinization (upper curve). The SE of the mean of 15 plates is included in the symbols.

PEs of untreated control cells remained essentially constant (~70%) for both exponential and plateau-phase cultures held in the various media. Similarly, the number of cells counted in each flask after various recovery times remained unchanged when compared to the number counted immediately after treatment, except in untreated flasks held in FM. In this case, the number of cells doubled by 24 hours.

Effect of Recovery Temperature

When exponentially growing cells were exposed for 1 hour at 37°C to 5 μg/ml of SHM, then allowed to recover in EBSS at different temperatures, the magnitude of recovery decreased as the temperature was lowered (fig 5). No recovery was observed at ambient temperature (~23°C), but an RF of ~4 was found at 30°C, while an RF of ~19 was observed at either 37°C or 40°C.

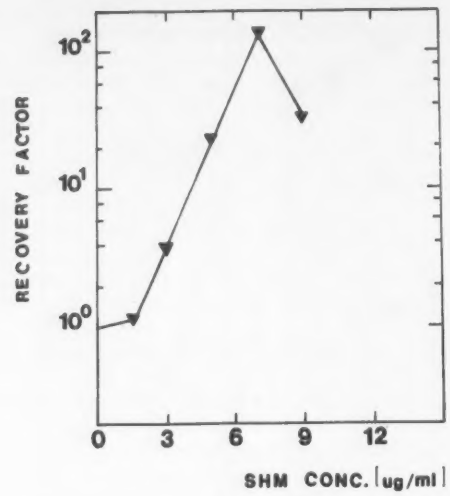

FIGURE 2.—Recovery expressed as RF for exponentially growing 9L cells treated for 1 hr with graded concentrations of SHM and held in EBSS at 37°C for 24 hrs before trypsinization. The SE of the mean of 15 plates is included in the symbols.

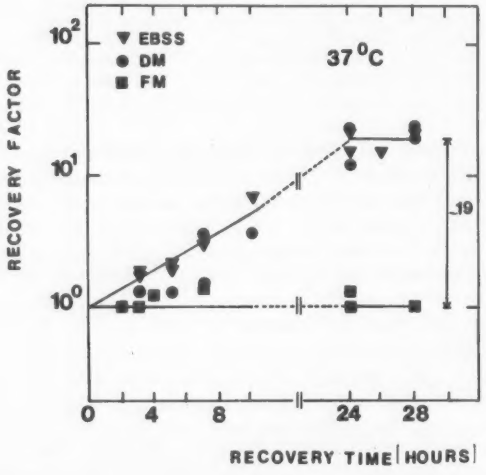

FIGURE 3.—Recovery expressed as RF for exponentially growing 9L cells treated for 1 hr with 5 μg/ml of SHM and held for various times before trypsinization in either EBSS, DM, or FM at 37°C. The SE of the mean of 15 plates is included in the symbols.

PEs of untreated control cells were ~70%, except for a recovery temperature of 40°C. In this case, the PE at 24–28 hours decreased to 50% of the initial value. For all temperatures the number of cells counted per flask immediately after treatment and after each recovery period remained constant (~3.5 × 10^6).

Effect of Cell Cycle Phase on Recovery (Cell Progression Analysis)

DNA profiles of untreated exponentially growing 9L cultures during the entire course of these experiments showed a substantial number of cells in G_1, S, and G_2 phases (fig 6). However, if these cells were held in FM for 24 hours after treatment (5 μg/ml of SHM for 1 hour at 37°C), most cells accumulated in middle to late S phase, with some accumulation in G_2. When either untreated or treated exponentially growing cultures were held in EBSS or DM, the number of cells at 24 hours in S and G_2-M phases decreased relative to untreated cells held in FM.

When control and treated plateau-phase cells were analyzed for DNA content immediately after SHM treatment, few cells were found in either S or G_2-M phase (fig 7). Therefore, most of the cells were arrested in G_1 phase. These DNA profiles remained unchanged when the cells were subsequently overlaid with either EBSS or DM for 24 hours. When these plateau-phase cells were held in FM, the control culture resumed cycling, and by 24 hours the DNA distribution was similar to that of exponentially growing cells. However, SHM-treated cells that were held in FM showed an accumulation of cells in mid to late S phase and a small proportion of cells in G_1 phase by 24 hours.

DISCUSSION

Recovery from PLD under suboptimal growth conditions such as DM and balanced salt solution and inhibition of recovery by FM have been widely observed after X-irradiation (6–8,11). Unlike results previously reported for bleomycin (5,10) and nitrosoureas (5), our data for SHM are in agreement with these observations.

Hahn (19) has suggested that cell recovery after insult can be analyzed in terms of the behavior shown by cells after damage induced by X-rays, ultraviolet (UV) rays, or a combination of both. The primary criterion for this classification was the half-life of recovery, which was ~2 hours for X-ray damage (19) and ~13 hours for UV damage (G. M. Hahn, personal communication). These half-lives sug-

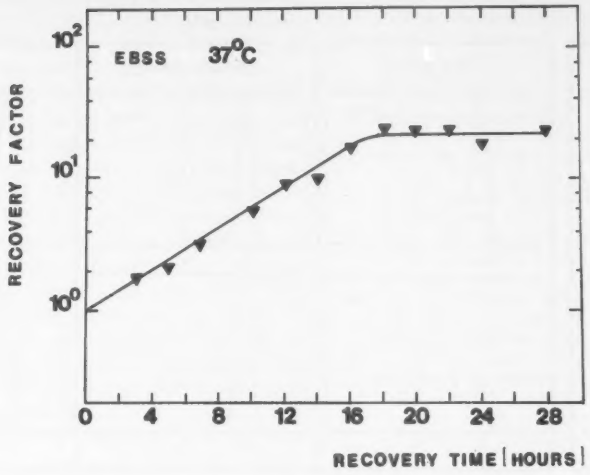

FIGURE 4.—Recovery expressed as RF for exponentially growing 9L cells treated for 1 hr with 5 μg/ml of SHM and held in EBSS at 37°C for various times before trypsinization. The SE of the mean of 15 plates is included in the symbols.

gested a repair mechanism acting at the DNA level, although the kinetics of these recovery processes and the kinetics of DNA repair after exposure to either X-rays or UV rays (20) were not identical. The kinetics of recovery from SHM-induced damage had a half-life of ~4 hours, and resembled the recovery observed after X-ray treatment.

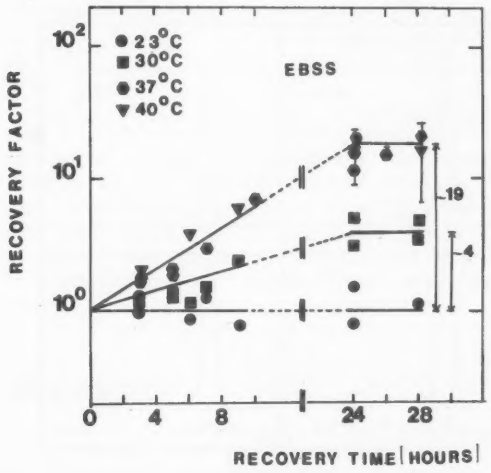

FIGURE 5.—Recovery expressed as RF for exponentially growing 9L cells treated for 1 hr with 5 μg/ml of SHM and held for various periods of time in EBSS at either 23°C, 30°C, 37°C, or 40°C before trypsinization. If not shown, the SE of the mean of 15 plates is included in the symbols.

The magnitude of recovery following SHM exposure was strongly dependent on drug concentration. The decreased enhancement of survival that resulted as the concentration was increased from 7 to 9 μg/ml might be related to the plateau region previously observed in the survival curve when 9L cells were treated with concentrations ≥ 9 μg/ml of SHM (4). However, this might also suggest that, at high concentrations, additional damage became fixed and thus lethal.

Our results show that the level of recovery after SHM treatment decreased at lower temperatures and was inhibited at ambient temperature (~23°C). This indicates that an enzymatic repair process was responsible for recovery. Similar temperature dependence of the recovery process was observed in X-irradiated HA-1 cells (11). In contrast, mouse L cells (12) and CHO cells (13) showed an enhancement of survival when cells were held at low temperature after X-ray treatment. Ross-Riveros and Leith (21) have reported that a 6-hour exposure to temperatures between 39°C and 41°C had no effect on 9L cell survival. In our experiments, the PE of untreated cells also remained at ~70% during the first 9 hours of exposure to EBSS at 40°C, but the decrease in PE observed in the control cultures by 24–28 hours indicated that prolonged exposure to mild hyperthermia is toxic for this cell line.

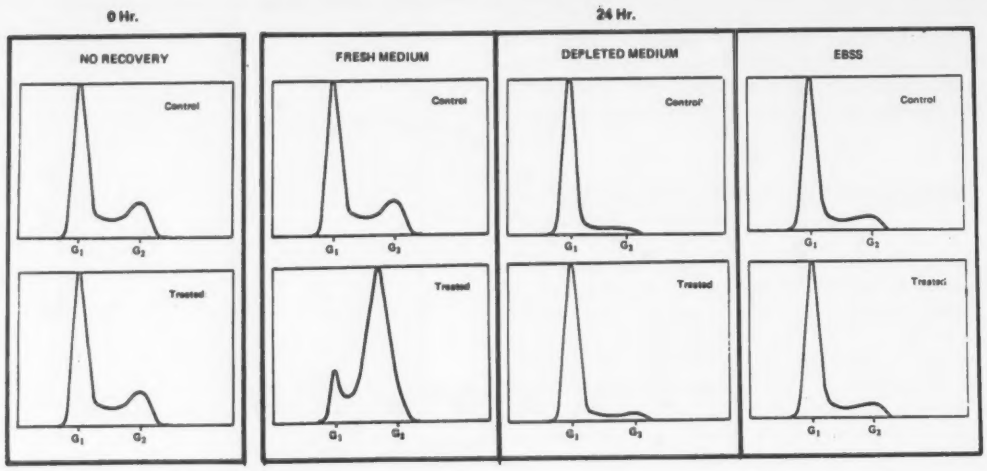

FIGURE 6.—DNA profiles of exponentially growing cultures treated for 1 hr with 5 μg/ml of SHM and either trypsinized immediately after treatment (no recovery) or held for 24 hrs in various media at 37°C before trypsinization. G_1 and G_2 indicate 2C and 4C DNA contents, respectively. The CVs of the G_1 peaks were ~5%.

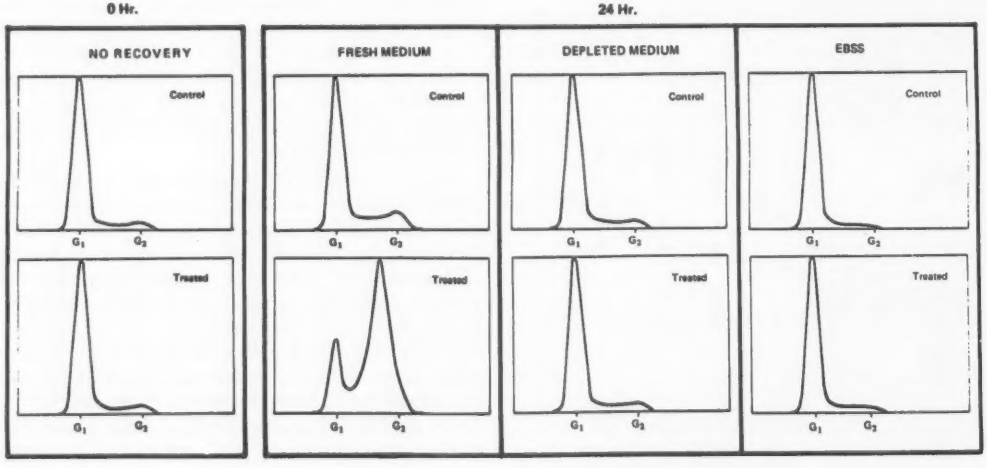

FIGURE 7.—DNA profiles of plateau-phase cultures treated for 1 hr with 5 μg/ml of SHM and either trypsinized immediately after treatment (no recovery) or held for 24 hrs in various media at 37°C before trypsinization. G_1 and G_2 have the same meaning as in fig 6. The CVs of the G_1 peaks were ~5%.

Earlier studies (4) showed that when cells were perturbed by SHM (*a*) the S, G_2, and M phases were prolonged; (*b*) there was a G_2 block; (*c*) some cells entered mitosis but few divided; and (*d*) cells tended to accumulate in mid-S, then moved synchronously to G_2-M, and remained there up to 72 hours. The rate at which cells moved was concentration-dependent and was much slower at high concentrations. Thus, the DNA histograms 24 hours after treatment with SHM show only one phase of the perturbation. However, the DNA histograms of cells that recovered from PLD were different from those of cells that did not recover from PLD. The results reported here show that incubation of exponentially growing or plateau-phase cultures in EBSS or DM arrested the cells in G_1, where they recovered from the drug damage. Conditions such as fresh medium that allowed cells to progress into S phase fixed damage and thereby reduced survival. These data are consistent with the results reported by Little (8) for human liver cells after X-ray treatment.

The results reported here may explain why single doses of SHM have not been effective against the 9L brain tumor in situ (4). If recovery from SHM-induced PLD also occurs in vivo, as already reported after X-ray (22) and bleomycin (10) treatment, tumor cells that recover would be able to contribute to the regrowth or the repopulation of the tumor. Because our preliminary studies have shown that 9L cells in vitro did not recover from damage induced by fractionated doses of SHM, fractionated administration of SHM may be more efficacious in vivo than treatment with a single dose of the drug.

Human brain tumors, which have many noncycling cells (23), may not respond to single doses of this agent. However, if the drug is administered after G_0 cells resume DNA synthesis, we might expect an inhibition of recovery from PLD and an increase in tumor cell-kill. Therefore, sophisticated drug scheduling in which SHM is administered after treatment with a cell cycle-non-specific agent may use the drug's characteristics to advantage.

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Studies on the Comparative Distribution and Biliary Excretion of Doxorubicin and 4′-Epi-doxorubicin in Mice and Rats[1,2]

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SUMMARY

Serum and tissue distribution of doxorubicin (DX) and its stereoisomer, 4′-epidoxorubicin (4′-epiDX), after iv injection of 15 mg/kg was investigated in C57BL mice bearing 14-day intramuscular Lewis lung carcinoma. Total fluorescence was measured together with unchanged drugs, separated, and quantitated by means of thin-layer chromatography combined with a scanning fluorescence technique. In serum the disappearance of 4′-epiDX paralleled that of DX and both unchanged isomers represented < 50% of the total fluorescence measured as early as 30 minutes after drug injection. In tissues the fluorescence measured was almost completely accounted for by the native compounds, both DX and 4′-epiDX, indicating that these drugs are taken up as such by tissues. 4′-EpiDX levels were markedly lower than those of DX in tumor and spleen, whereas in heart, liver, and kidneys the concentrations of the two isomers were the same. Traces of doxorubicinol and possibly 4′-epidoxorubicinol were detected only in serum, liver, and kidneys. Comparative cumulative biliary excretion of DX and 4′-epiDX investigated in the rat indicated that a total of 40%–45% of the injected dose of both drugs was excreted either as unchanged compound or as reduced metabolite. However, the proportions were different and the presence of twice as much reduced metabolite and smaller amounts of native 4′-epiDX suggests that its metabolic rate is different from that of DX.

[Cancer Treat Rep 64:897–904, 1980]

The widely used antitumor antibiotic, doxorubicin (DX), is limited in clinical use by its toxicity, in particular, acute myelotoxicity and severe cardiomyopathy (1). Reports of DX analogs with less toxicity but equal antitumor activity are therefore of considerable interest.

Among the recently synthesized glycoside derivatives of anthracyclines, the 4′-epi analogs of DX and daunorubicin (DR) differ from the parent drug in having the hydroxyl group at C-4′ of the aminosugar equatorial instead of axial (2) (fig 1); they are reported to have biologic activity similar to that of the parent drugs on different cultured cell lines and in several experimental tumor systems (2,3). It has also been shown (4,5) that in vitro inhibition of mouse embryo fibroblast proliferation, binding to DNA, and inhibitory activities on DNA synthesis are not affected by 4′-epimerization of DX and DR. The different orientation of the C-4′-hydroxyl may, however, result in some differences in pharmacologic behavior. It was found that 4′-epidoxorubicin (4′-epiDX), at concentrations at which DX is very effective, does not inhibit the beating rate of cultured heart cells although its antineoplastic activity

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is comparable to that of DX in animal tests (2), with lower toxicity.

These observations suggest the potential therapeutic interest of 4′-epimers of DX and DR which are currently under clinical investigation (phase I) (6–8) and prompt further studies to elucidate possible pharmacologic differences from their parent compounds. For instance, altered cell pharmacodynamics was found by Di Marco et al (3) who reported that the modification introduced in the amino sugar by the 4′-epiconfiguration results in different basicity of the DX and DR molecules, which should alter membrane binding and transport. Indeed, these DR and DX analogs, being weakly ionized, could be distributed in the body differently from the reference compounds; in fact, they show a higher rate and degree of accumulation in the L1210 cells than the parent drug.

The present paper describes a comparative study of plasma and tissue distribution of the two epimers, DX and 4′-epiDX, in an experimental mouse tumor, the Lewis lung carcinoma, and gives data on their biliary excretion, the main excretory pathway for DX and metabolites in a variety of species (9,10). Only the α anomers of these DX stereoisomers have been considered since the inverted configuration at position 1 of the aminosugar reportedly has much less biologic activity (4).

MATERIALS AND METHODS

Animals and Tumor

C57BL/6J male mice (body weight, 22 ± 3 g) and CD-COBS male rats (body weight, 200 ± 20 g) obtained from Charles River Breeding Laboratories, Italy, were used for these experiments. The mice received an im transplant of 2×10^6 viable cells of Lewis lung carcinoma maintained by im passages in the same strain every 15 days.

Drugs

DX and its analog 4′-epiDX were obtained from Farmitalia-Carlo Erba, Milan, Italy. For pharmacokinetic studies, DX and 4′-epiDX were injected iv at a dose of 15 mg/kg in mice bearing 14-day intramuscular Lewis lung carcinoma; 1, 5, 15, 30, and 60 minutes and 3, 6, 16, 24, and 48 hours after the injection, five animals per point were killed and serum and tissues (tumor, heart, spleen, kidneys, and liver) were removed and frozen at –20°C until use.

CD-COBS male rats were used for determination of drug biliary excretion. Rats were anesthetized with ethylurethane (25%) and the bile duct was isolated and cannulated. DX and 4′-epiDX were injected iv at a dose of 10 mg/kg in four rats per group and bile was collected continuously for 30 hours after treatment. Bile samples were stored at –20°C.

Biochemical Assay of DX and 4′-EpiDX

Total DX and 4′-epiDX fluorescence was measured in serum after extraction and deproteinization with *n*-butanol for DR as described by Finkel et al (11). At an activation wavelength of 475 nm and an emission of 580 nm, recovery was 90% and sensitivity was 25 ng/ml. Adaptation to tissues was performed for both drugs by extracting with *n*-butanol after deproteinizing with $AgNO_3$, according to Schwartz (12) with slight modifications. Under these conditions recovery was 70% and sensitivity was 0.5 μg/g. Since these methods do not differentiate between native drugs and their metabolites, the concentrations are expressed as DX and 4′-epiDX equivalents.

To separate and quantify the unchanged drugs and their reduced metabolites, doxorubicinol (DXol) and 4′-epidoxorubicinol (4′-epiDXol), samples of plasma, tissues, and bile were submitted to thin-layer chromatography (TLC) and quantified by a fluorescence scanning technique (CAMAG-TLC scanning accessory attached to a Turner model fluorometer Model 111) (13,14). Samples of serum were extracted once with 2 ml of ice-cold chloroform:isopropanol (1:1) and the resulting gel was mixed with sufficient solid ammonium sulphate to give a saturated solution. After centrifugation at 4000 rpm the organic phase was transferred to a 10-ml Pyrex tube and evaporated under vacuum to dryness. Samples of tissues were deproteinized with $AgNO_3$ (33%) and extracted with 5 ml of ice-cold chloroform:isopropanol (1:1); 4.5 ml of the organic phase was transferred to a 10-ml Pyrex tube and evaporated under vacuum to dryness.

The serum or tissue residues were re-dissolved with 50 μl of a chloroform:methanol (1:1) solution containing DR as internal standard. The entire samples were spotted on silica gel plates (DC-Alufolien Kieselgel 60, 0.2 mm thick, Merck). Bile was applied directly to the thin-layer plates for chromatographic analysis. Samples and extracts were separated in an ascending fashion, first for 16 cm in pure ethylacetate, then for 12 cm in chloroform:methanol:acetic acid:water (80:20:14:6). The plates were dried briefly and allowed to stand 10 minutes before scanning. An activation wavelength of 475 nm and an emission of 580 nm were used for quantification of DX, 4′-epiDX, and reduced me-

tabolites; the concentration of each fluorescent spot was determined from the ratio of the fluorescent areas of standard drug solutions to the DR area. Since no 4′-epiDXol standard was available, the spot on the TLC from 4′-epiDX-treated mice with an R_f value corresponding to the DXol spot was considered to be 4′-epiDXol.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic parameters in plasma were calculated by mathematically analyzing the results with the peeling method assuming a three-compartment open model (15).

For tissues, the areas under the concentration versus time curves (AUC) were measured by trapezoidal integration and the half-life by calculation of the significance of the regression using the determination coefficient r^2 (least-squares method) (16).

RESULTS

The disappearance curve of DX and its stereoisomer, 4′-epiDX, in the serum after iv injection of 15 mg/kg in mice bearing 14-day intramuscular Lewis lung carcinoma is shown in figure 2. The disappearance rates of 4′-epiDX parallel its epimer DX, although the levels of total fluorescent compounds or of the unmetabolized drug are somewhat lower than those of DX. In addition, both native isomers represent < 50% of the total fluorescence measured in serum as early as 30 minutes after drug injection. The reduced metabolites, DXol and 4′-epiDXol, were detectable, but the concentrations were too low for quantitative measurement.

Pharmacokinetic analysis according to a three-compartment open model which, under our conditions, resulted to fit the experimental findings better than a two-compartment model either for total fluorescence or for unchanged compounds showed no substantial differences between DX and 4′-epiDX in the parameters Co, half-lives (alpha, beta, and gamma), AUC, and clearance (table 1). Compared to the corresponding total fluorescence, both unchanged drugs show marked lower AUC, as expected, on the basis of the experimental results, shorter beta half-life, and higher clearance, accounted for by better elimination from the circulation. The longer gamma half-life may suggest strong protein binding, impaired entrance into poorly perfused tissues, or subsequent re-distribution into the bloodstream.

The distribution of DX and 4′-epiDX to the different tissues (tumor, spleen, heart, liver, and kidneys) of Lewis lung carcinoma-bearing mice injected with the same drug dose of 15 mg/kg (fig 3 and table 2) shows that, in contrast with the serum compartment, the disappearance curve of unchanged DX and 4′-epiDX from all tissues studied is superimposable to that of the corresponding total fluorescence (peak levels, AUC, and half-lives differ by ≤ 25% for the two analogs). Only in liver, and possibly in tumor, the levels of native 4′-epiDX seem lower than the concentrations of total fluorescence measured. The reduced metabolites of

TABLE 1.—Serum pharmacokinetic parameters of DX and 4′-epiDX (15 mg/kg iv) in mice bearing 14-day intramuscular Lewis lung carcinoma*

		Curve parameters†				
	Co (μg/ml)	Half-life (mins)	Exponential segment slopes	Ordinate intercepts	AUC (μg/ml × min)	Clearance (liter/kg/min)
DX equivalents	42.9	α 1.5	a 0.466	A 42.9	911	0.016
		β 113	b 0.0061	B 1.36		
		γ 485	c 0.0014	C 0.86		
DX	34.1	α 2.0	a 0.356	A 34.1	366	0.041
		β 66	b 0.0118	B 0.737		
		γ 753	c 0.0011	C 0.220		
4′-EpiDX equivalents	19.6	α 1.8	a 0.379	A 19.6	781	0.019
		β 100	b 0.0069	B 1.828		
		γ 525	c 0.0013	C 0.612		
4′-EpiDX	20.8	α 1.8	a 0.389	A 20.8	302	0.050
		β 57	b 0.0121	B 1.588		
		γ 745	c 0.0009	C 0.114		

*Pharmacokinetic analysis by the peeling method assuming a 3-compartment open model.

†The equation describing the curve is: $Cp = Ae^{-at} + Be^{-bt} + CE^{-ct}$, where Cp = plasma concentration at time t, A, B, C = intercepts on the ordinate at 0 time, and a, b, c = the slopes of the respective exponential segments.

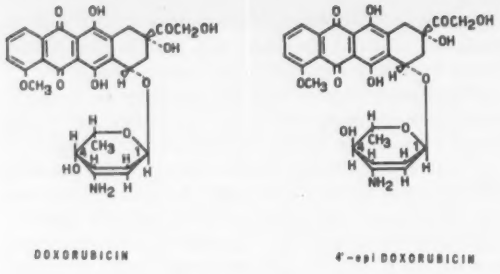

FIGURE 1.—Chemical structure of the 2 anthracycline isomers.

both isomers, DXol and possibly 4'-epiDXol, were found (besides in serum) only in the liver and kidneys, but again the levels were too low to allow quantification. Within the sensitivity limits of the method no reduced metabolites were detectable in heart, spleen, and tumor. With regard to the levels of the two analogs in different tissues (where peak concentrations were already attained 30 minutes after drug injection), the pattern of distribution was very similar (table 2). Concentrations measured were comparable for both compounds in the heart, liver, and kidneys; only in tumor and more clearly in spleen were considerably lower amounts of the 4'-epi isomer found (peak levels and AUC of DX in the spleen were twice as high as 4'-epiDX).

The hepatobiliary metabolism and elimination of DX and 4'-epiDX were investigated in the rat model where the bile flow is continuous, because of the lack of a gallbladder. Since it has been described (17) that DX accounts for > 80% of total drug fluorescence excreted in the bile and that one half of the remainder is represented by the major metabolite, DXol, it was considered of interest to compare the cumulative biliary excretion of the unchanged isomers and their reduced metabolites DXol and 4'-epiDXol (fig 4).

Of the total DX dose, 19.6% at 8 hours and 28.6% at 30 hours were recovered in the bile as unchanged compound, compared to only 13.2% and 20% recovered at the same times as unchanged 4'-epiDX.

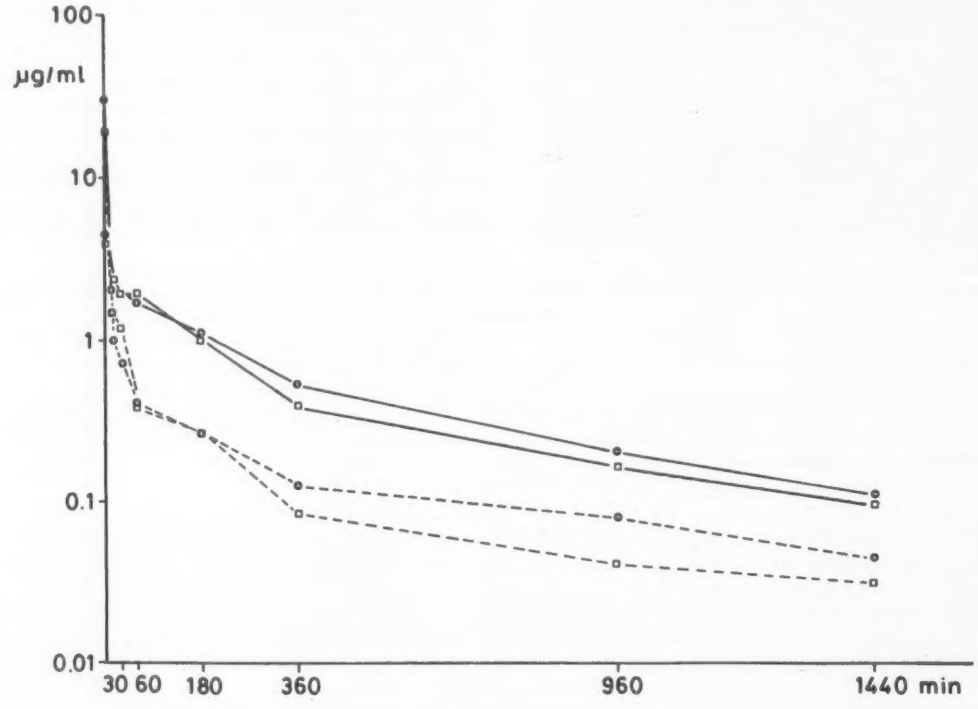

FIGURE 2.—Disappearance curve of DX and 4'-epiDX in serum of mice bearing intramuscular Lewis lung carcinoma (14-day) after iv injection of 15 mg/kg. O———O = DX equivalents; O----------O = DX; □———□ = 4'-epiDX equivalents; □---------□ = 4'-epiDX.

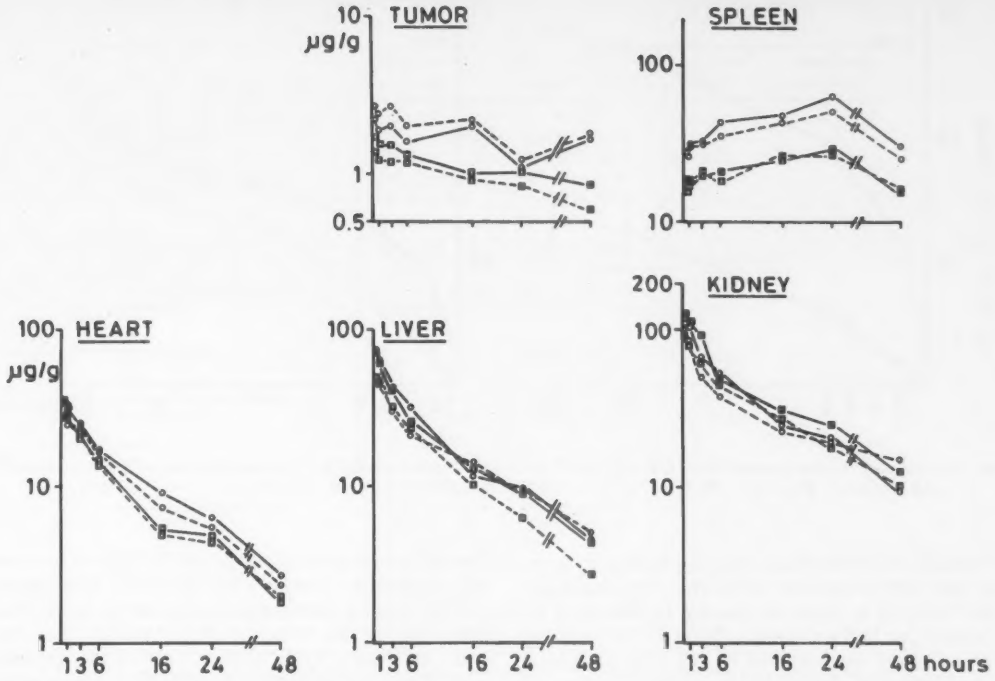

FIGURE 3.—Disappearance curves of DX and 4′-epiDX (15 mg/kg iv) in tissues of mice bearing intramuscular Lewis lung carcinoma (14-day). O——O = DX equivalents; O---------O = DX; □——□ = 4′-epiDX equivalents; □---------□ = 4′-epiDX.

However, at 30 hours the reduced metabolite accounts for only 13.3% of the injected dose of DX as compared to 19% of the 4′-epiDX dose, indicating that 4′-epiDX is metabolized more extensively by the enzyme aldoketoreductase. Peaks revealing the presence of more polar metabolites were found, but it was difficult to quantify them in our experimental conditions.

DISCUSSION

The findings described here indicate that epimerization at position 4′ has only a limited effect on the serum pharmacokinetics of DX, although the levels of 4′-epiDX were somewhat lower than those of DX. As observed for this antitumoral antibiotic (14,18), shortly after drug injection the unchanged 4′-epimer of DX represents much less than 50% of the total fluorescence measured, suggesting that the compound is very rapidly cleared by metabolism. The parallelism between the serum disappearance curves of 4′-epiDX and its parent compound DX is well-documented by the pharmacokinetic parameters calculated, which were very similar. Native DX and its 4′-epi analog seem to be distributed to the nonblood compartment, at least to the highly perfused tissues, to a greater extent than total fluorescence and cleared from the bloodstream better and more rapidly (two to three times higher clearance, shorter beta half-life). The gamma phase, which should describe drug redistribution from tissues to blood, is characterized by a longer half-life, indicating longer persistence of the native drugs in the circulation probably bound to proteins. This might account for a subsequent release from tissues of the unmetabolized form of the two epi analogs, which is in good agreement with their high uptake by tissues (80%–90% of the fluorescence measured is unchanged drug).

The rapid clearance of DX from plasma and its considerably extensive deposit in tissues has been reported in previous studies (19–21), and is confirmed by our results. 4′-EpiDX seems to behave the same way and to reach the same levels as DX in tissues, except in spleen and possibly in tumor, where 4′-epiDX concentrations were lower. These findings are in agreement with the report by Casazza et al (22), who described the tissue distribution

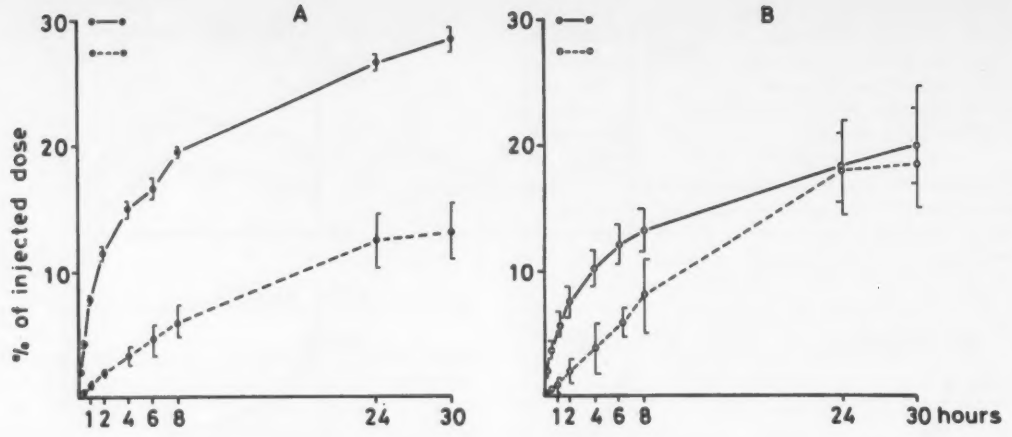

FIGURE 4.—Cumulative biliary excretion of DX and DXol (A), 4′-epiDX and 4′-epiDXol (B) in rats injected with DX or 4′-epiDX (10 mg/kg iv). ●——● = DX; ○——○ = 4′-epiDX; ●----● = DXol; ○----○ = 4′-epiDXol.

of 4′-epiDX by estimating total fluorescence; however, they are in contrast with what was expected on the basis of in vitro studies by Di Marco et al (3), where the higher uptake of the DX isomer by tumor cell lines as compared to DX itself suggests the possibility of greater in vivo accumulation of this compound in the tumor tissue.

Since the drug concentration at the target site is considered one of the determinants of drug response, and since the two analogs have been reported to display similar cytotoxic activity (2,3), the fact that the concentrations of 4′-epiDX found in spleen are 50% lower than DX could have some significance in relation to the purportedly lower toxicity of the 4′-DX epimer (2,3). In the scope of ongoing research aiming at investigating the hematologic and immunologic toxicity of the two analogs, unpublished data from this laboratory indicate that the spleen weight, which is markedly decreased by DX, is unaffected by 4-epiDX treatment; moreover, when 4′-epiDX is given, leukopenia appears significantly lower as compared to that after administration of DX. In dealing with the problem of cardiotoxicity and the stimulating report that unlike DX, 4′-epiDX did not affect the beating rate of cultured heart cells (2), our findings of equivalent concentration of the two drugs in the heart do not bear out the assumption of different pharmacologic behavior. Moreover, as described for rabbits (21) and mice (23), DX concentrations are not excessive or unusually high in the rat heart either; the AUC after 24 hours is two to four times lower than in other tissues. It is thus reasonable to assume that the cardiotoxicity is more probably related to a specific sensitivity than to avid uptake by this tissue.

DX, which is poorly excreted in urine, is excreted primarily by the biliary route in rabbit, dog, rat, and man (18,21,24,25), and its main metabolic pathway is biotransformation to DXol by means of intracellular cytoplasmic aldoketoreductase (26). DX, however, seems to be a poor substrate for aldoketoreductase in the rat, since Israel et al (17) found that > 80% of total fluorescence recoverable in the bile was unchanged compound and that only approximately 4% of the dose administered was DXol.

Although our findings show a higher production of reduced metabolite (13% of the dose was found as DXol in the bile during 30 hours), they substantiate the inability of other investigators to measure DXol quantitatively in serum or tissues of treated rats. In other species, such as rabbits, however, the rate of biotransformation to DXol is different; they excrete twice as much DXol as unchanged DX in the bile, and have significant quantities of the reduced metabolite in serum and tissues (21). In man the levels of this metabolite in serum are comparable to those of native DX (14).

A quantitatively different metabolic rate is also found for 4′-epiDX, which undergoes greater carbonyl reduction to 4′-epiDXol; 18% of the dose excreted in the bile is represented by this metabolite, the total percentage of unchanged compound and reduced metabolite excreted via the biliary route being similar to that for DX (~40%). Enhanced biotransformation of the 4′-epimer of DX is further suggested by the lower levels of native drug meas-

TABLE 2.—Tissue levels of DX and 4′-epiDX (15 mg/kg iv) in mice bearing 14-day intramuscular Lewis lung carcinoma

Tissue	Peak level (μg/g)	AUC after 48 hrs (μg/g × min)	Half-life* (hrs)	Reduced metabolite†
Liver				
DX equivalents	75.7 ± 8.4	44074 ± 3611	12.0	
DX	51.5 ± 7.2	38259 ± 3319	14.8	+
4′-EpiDX equivalents	72.1 ± 5.9	41342 ± 3702	12.3	
4′-EpiDX	47.4 ± 4.7	31939 ± 3010	11.4	+
Kidney				
DX equivalents	116.1 ± 11.0	79975 ± 8034	13.6	
DX	101.9 ± 8.0	73069 ± 7805	19.2	+
4′-EpiDX equivalents	131.8 ± 10.4	94942 ± 9183	14.7	
4′-EpiDX	93.6 ± 3.1	74943 ± 7065	15.2	+
Heart				
DX equivalents	33.4 ± 1.7	26649 ± 2454	13.2	
DX	27.3 ± 3.7	23282 ± 2328	12.9	–
4′-EpiDX equivalents	33.1 ± 1.6	20291 ± 1692	11.6	
4′-EpiDX	34.4 ± 3.4	20477 ± 2610	12.1	–
Spleen				
DX equivalents	62.4 ± 3.5	131571 ± 9950	ND	
DX	51.3 ± 2.5	111280 ± 4890	ND	–
4′-EpiDX equivalents	29.2 ± 3.1	65158 ± 4921	ND	
4′-EpiDX	27.3 ± 3.4	63929 ± 5865	ND	–
Tumor				
DX equivalents	2.2 ± 0.3	4679 ± 638	ND	
DX	2.8 ± 0.3	5345 ± 806	ND	–
4′-EpiDX equivalents	1.7 ± 0.3	3214 ± 388	ND	
4′-EpiDX	1.5 ± 0.4	2537 ± 366	ND	–

*Calculation of half-life by the least-squares method (16). ND = not determined because drug levels were still at a plateau 48 hrs after administration.

†Presence or absence of the reduced metabolite. DXol or 4′-epiDXol was indicated by + or –, since the amount of metabolite, even if present, was too low for quantification.

ured in the liver as compared to the total fluorescence, in contrast to what is observed for DX.

As to whether the different metabolic rate observed with the two epimers is involved in modifications of response, unpublished in vitro studies from this laboratory show that the reduced metabolite of these compounds is always much less active than the parent drug; therefore, the 5% increased production of reduced metabolite with 4′-epiDX treatment should have a slight relevance in terms of activity.

In conclusion, it seems that epimerization at position 4′ may modify the metabolic fate of DX and in some way its pharmacokinetics. What relevance the lower distribution to the spleen and the altered metabolization of this 4′-epi compound has in terms of pharmacologic behavior could be a problem worthy of further investigation.

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Phase I Study of *cis*-Diamminedichloroplatinum(II) Administered as a Constant 5-Day Infusion[1]

Jacob J. Lokich[2,3,*]

SUMMARY

Thirty patients with metastatic malignancy of various types were treated with *cis*-diamminedichloroplatinum(II) (DDP) administered by continuous infusion for 120 hours. The starting dose was 20 mg/m²/day (100 mg/m²/course) and was escalated by stages to 40 mg/m²/day (200 mg/m²/course). Dose-limiting toxicity was observed at 30 mg/m²/day (150 mg/m²/course), manifested as marrow suppression and particularly thrombocytopenia in 13 of 14 patients evaluated at doses ≥ 30 mg/m²/day. The gastrointestinal toxicity characteristic of bolus treatment schedules was less intense but was cumulative and dose-related. Renal toxic effects developed in five of 30 patients in spite of adequate hydration and daily diuretic therapy. Peripheral neuropathy developed in the only two patients who received four courses of continuous-infusion DDP. Antitumor effects were observed in six patients (oral cancer, two; lymphoma, one; prostatic cancer, one; hepatoma, one; and bronchogenic carcinoma, one). The recommended starting dose for continuous venous infusion therapy with DDP is 30 mg/m²/day for 5 days.

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cis-Diamminedichloroplatinum(II) (DDP) is one of a group of platinum compounds which has demonstrated clinical activity in a broad spectrum of malignancies including testicular, ovarian, bladder, and head and neck cancers (1,2). In preclinical animal studies schedule dependency was not demonstrated and in clinical studies the drug schedule generally employed has been bolus or short-term infusion on Day 1 or daily for 5 days with the course repeated at 3–4-week intervals (3,4).

Prolonged constant infusion of a cytotoxic agent may allow for drug exposure to all cells which subsequently enter cycle, as opposed to the short term treatment by bolus which allows only a random proportion of cells that are in cycle at that point to be affected. Drewinko et al (5), in an in vitro system, have demonstrated that DDP kills tumor cells comparably in all stages of the cell cycle but also suggested that an increased antitumor effect with an improved therapeutic index is obtained by low-dose infusion. Therefore, a constant-infusion study of DDP administered for 120 hours (5 days) was undertaken to determine the maximally tolerated dose and the qualitative patterns of toxicity and to compare the toxicity with that observed with bolus injection.

MATERIALS AND METHODS

All patients entered in the continuous-infusion DDP trial had a histologic diagnosis of metastatic malignancy and were considered candidates for investigational therapy in that standard forms of therapy were exhausted or not established. Patients had a life expectancy of > 4 weeks and pa-

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tients who had received prior radiation therapy or chemotherapy had a minimum lapse of 4 weeks before entry in the DDP trial. All patients had renal scan or iv pyelogram to determine the patency of the urinary tract, and 24-hour urine creatinine clearance was determined to evaluate renal function reserve. Initial entry in the study required the presence of two kidneys with patency of the ureters and a minimum creatinine clearance of 80 ml/minute. Subsequently, these stringent criteria for entry in the study were modified; four patients were entered with anatomic renal compromise (solitary kidney, two patients; unilateral obstructed ureter, two patients), and the creatinine clearance minimum was decreased to 60 ml/minute.

Prior to initiation of drug infusion, patients were hydrated with 1000 ml of 5% dextrose in one-half normal saline to which 20 mEq of KCl was added. The pretreatment hydration period ranged from 5 to 10 hours and at Time 0, 40 mg of furosemide was administered either orally or iv. The DDP infusion was administered in two or three liters of 5% dextrose in one-half normal saline with 20 mEq of KCl added to each liter. The drug was distributed in the infusate equitably in the projected volume and infused by constant pump infusion for 5 consecutive days.

Patients were encouraged to drink an additional 2 liters daily and intake and output were monitored to ensure an output of > 2 liters. Creatinine clearance was tested over the last 24-hour period of infusion. Furosemide was administered on a daily basis for each of the 5 consecutive days.

Subsequent to the treatment course patients were monitored on a weekly basis by cbc and platelet count as well as by serum creatinine, and tumor parameters were monitored at monthly intervals. Therapy was repeated at 4-week intervals if no tumor growth was observed in the interim and if drug tolerance was optimal. Patients were evaluated daily during drug administration for gastrointestinal effects of the drug. Gastrointestinal toxicity was graded as follows: 1 = transient nausea without anorexia; 2 = intermittent vomiting with meals only; and 3 = protracted daily vomiting. For the last five patients entered in the study, pretreatment serum magnesium levels were compared to Day-5 serum magnesium levels.

Standard definitions of tumor response were used. Partial response was defined as a > 50% decrease in the product of the perpendicular diameters of all discretely measurable lesions. Complete response was defined as a complete regression of all measurable disease for a minimum of 1 month with symptomatic improvement.

TABLE 1.—Frequency and patterns of acute toxicity of constant-infusion DDP

Organ toxicity	No. of DDP courses	% of total courses producing toxic effects
Gastrointestinal*		
Grade		
1	5	
2	9	24
3	1	
Ototoxicity	3	7
Neuropathy*	2	
Renal	5	11
Marrow suppression	13	32

*Gastrointestinal and neurologic toxic effects were observed as cumulative effects (*see* text).

RESULTS

A total of 30 patients (15 females and 15 males) were entered in the study to receive continuous-infusion DDP. The median age at the time of treatment was 55 years, with a range of 19–80 years. Thirteen patients had received prior chemotherapy and six patients had received prior radiation therapy. Twenty-four of the 30 patients had a performance status of 0–1 (Eastern Cooperative Oncology Group scale). The tumor categories represented included lung cancer (seven patients: two with adenocarcinoma, two with epidermoid carcinoma, and three with undifferentiated carcinoma); pancreatic cancer (five); ovarian cancer (four); head and neck lesions (three); prostatic and breast cancer (two each); and one each of melanoma, gastric cancer, vaginal cancer, renal cell carcinoma, hepatoma, lymphoma, and a tumor of unknown origin. Twenty-five of the 30 patients had measurable disease evaluable for response and were followed for a minimum of 1 month.

The pattern of toxicity observed with constant-infusion DDP differed from that observed when the drug is administered by bolus or short-term infusion (table 1). The most striking difference was the dramatic reduction in gastrointestinal toxicity. Only ten of 41 courses were associated with grade 2 or 3 emesis and only at doses of > 30 mg/m²/day. Five patients had nausea only but this was generally an effect associated with eating. In the patients with grade 2 or 3 gastrointestinal toxic effects, a conditioning phenomenon was the rule; toxicity was accentuated with subsequent courses. Within courses, the gastrointestinal toxicity progressed over the 5-day treatment period.

Ototoxicity, observed in three patients, was transient and manifested primarily as tinnitus which

TABLE 2.—Frequency of renal failure (creatinine > 1.5 mg/dl) and marrow suppression in relationship to dose

DDP dose (mg/m^2)	No. of patients	No. of courses	No. of renal failures/ No. of patients	Marrow suppression (No. of cases/No. of patients)	
				Platelets < 100,000/mm^3	Wbcs < 2500/mm^3
20	14	15	1/14	0	0
25	10	10	0	0	1/10
30	6	6	0	6/6	2/6
35	4	5	2/4	3/3*	2/3*
40	5	5	2/5	4/5	4/5
Totals	39	41	5/39	13/38	9/38

*Denominator denotes only patients who had weekly cbcs.

disappeared after drug withdrawal. One of the three patients had had a prior stapedectomy; that patient developed tinnitus on Day 4 of the 5-day infusion.

Renal toxic effects were observed in five patients (with the initial course in four of the five). All patients had adequate hydration and urinary outputs throughout the DDP infusion. The maximum serum creatinine level was 3.2 mg/dl, and in all patients renal function recovered. In two patients with transient renal failure a second course of DDP led to the recurrence of renal failure which also was transient. The renal effect was apparently dose-related in that four of the five incidents developed at 35 or 40 mg/m^2/day.

A peripheral neuropathy with generalized (constitutional) and proximal muscle weakness and areflexia was observed in the two patients who received four courses of DDP. This neuropathy, similar to the gastrointestinal toxic effects, appeared to be a cumulative effect of the DDP.

A bone marrow effect was observed at doses beyond 30/mg/m^2/day or 1½ times the standard total dose per course (table 2). Thirteen of 14 patients developed thrombocytopenia with or without leukopenia and ten events were considered life-threatening in that the platelet count was < 50,000 or the wbc count was < 2000/mm^3. The marrow suppression episodes were always self-limited and without associated hemorrhage, sepsis, or mortality. In fact, none of the patients required hospitalization for thrombocytopenia nor did they require platelet transfusions. The cell count nadir for thrombocytopenia occurred at approximately Day 10 and for leukopenia it occurred approximately 1 week later, with complete recovery by Day 21–28. Anemia was a prominent apparent complication of therapy although the underlying disease may have accounted for ineffective erythropoiesis superimposed on the drug effect. At least four patients required blood transfusions for symptomatic anemia. Two patients had pancytopenia prior to treatment due to marrow replacement by tumor but the pancytopenia was not accentuated.

Responses were observed in six of the 25 evaluable patients: one patient each with prostatic cancer, non-Hodgkin's lymphoma, hepatoma, and undifferentiated lung cancer, and two patients with head and neck cancers. The latter two patients continued to receive therapy, but two of the former patients developed transient renal failure (one patient) and progressive disease (one patient) necessitating discontinuation of DDP therapy. No responses were observed in six patients with lung cancer or in five patients with pancreatic cancer. Two of the four patients with ovarian cancer were evaluable and neither responded to therapy after failing treatment with a standard alkylating agent. All responses were observed at doses of 20–30 mg/m^2/day.

DISCUSSION

The rationale for continuous or constant-infusion chemotherapy is provided by cytokinetic and pharmacologic considerations. Cytokinetically, Sigdestad et al (6) have observed that DDP preferentially kills during the G_1 phase although the drug is active in all phases of the cell cycle. Constant infusion theoretically allows for exposure to a quantitatively greater number of G_1 cells. Pharmacologically, DeConti et al (3) have established that DDP has a biphasic mode for plasma clearance with a half-life in the first phase of 25–49 minutes and in the secondary phase of up to 3 days. In their studies, albumin binding was prominent in the secondary phase. DDP pharmacology is comparable for a bolus and infusions up to 48 hours with probable accumulation of drug in the short-term constant-infusion groups (7). Although the pharmacologic data do not

support a rationale for continuous-infusion programs, studies of DDP delivered by extended constant infusions have not been reported. Jacobs et al (8) studied 24-hour infusion of DDP and Salem et al (9) reported on 53 patients treated with 120-hour infusions administered alone or in combination with other drugs. In the latter study, the patterns and frequency of toxicity were greatly diminished over that observed with bolus injection. Yap et al (10) studied 12 patients treated with continuous-infusion DDP at a dose of 20 mg/m²/day. In comparison to bolus DDP gastrointestinal effects were less frequent but thrombocytopenia was more severe with the continuous-infusion delivery schedule. In none of these studies was dose escalation undertaken.

Dose-limiting toxicity for DDP administered by continuous infusion is marrow suppression as well as renal and gastrointestinal toxic effects. Gastrointestinal toxicity of grade 2 severity was experienced by most patients at the higher dose levels of 35–40 mg/m²/day by Day 4 or 5 of the constant infusion, but in only one course was grade 3 toxicity observed. The hematopoietic toxicity was consistent in terms of inducing anemia in a majority of the patients and was comparable to the 60% incidence of anemia and transfusion requirement observed in the study by Salem et al (9). Life-threatening hematopoietic toxicity in the form of thrombocytopenia was dose-related, and 75% of the thrombocytopenic episodes were at doses of > 30 mg/m²/day.

Nephrotoxicity and renal magnesium wasting due to platinum have been previously reviewed (11,12). Nephrotoxicity was observed in five of 41 courses (11%) and the clinical characteristics were consistent with previous studies. Four of the five incidents occurred at doses of > 30 mg/m²/day suggesting a dose relationship.

The therapeutic index for DDP is improved by constant-infusion drug administration as a consequence of the resulting decrease in acute gastrointestinal toxicity; whether or not cytotoxicity or tumor cell-killing is increased is not yet determined. Dose-limiting toxicity was observed at 30 mg/m²/day (total, 150 mg/m²) with thrombocytopenia in all patients but the platelet count nadir was life-threatening (< 50,000/mm³) in only 50%. At 35 and 40 mg/m²/day (cumulative dose, 175–200 mg/m²) renal failure and gastrointestinal toxic effects were observed at unacceptable frequency and the marrow suppression was also more commonly seen at life-threatening thrombopenic levels. The recommended dose for good-risk patients (ie, no prior therapy; < 60 years of age; and no bone or bone marrow disease) is 30 mg/m²/day and for poor-risk patients it is 25 mg/m²/day.

In this phase I trial isolated responses were observed in tumors for which DDP has identified activity. There was no demonstrated activity in patients with lung and pancreatic cancers which are resistant to DDP in studies employing bolus or short-term infusion. The expanded phase II trial of constant-infusion DDP will focus on DDP-resistant or -insensitive tumors including melanoma, gastrointestinal cancer, breast cancer, and mesenchymal tumors. Combination programs of constant-infusion DDP with drugs that are non-cross-resistant and non-complementary with regard to toxicity will also be explored in DDP-sensitive tumors such as ovarian, bladder, head and neck, and testicular cancers.

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Cytotoxic Chemotherapy in Adrenal Cortical Carcinoma[1,2]

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SUMMARY

Adrenal cortical carcinoma is a rare tumor with a poor prognosis. Experience with chemotherapy in this tumor besides mitotane is practically nonexistent. We are reporting the results of 27 trials of various chemotherapeutic regimens among 12 patients with metastatic disease. Most patients (nine of 12) had a trial of mitotane before cytotoxic drugs were instituted. Three patients achieved a partial remission, one achieved a minor regression, and three had stable disease. All of the responses were of short duration. Alkylating agents and doxorubicin appear to have some antitumor activity. Multi-institutional cooperative studies are needed to detect active drugs and drug combinations against this neoplasm.

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Adrenal cortical carcinoma is a rare malignant neoplasm with an incidence of two per million population (1–3). Surgery is the treatment of choice for localized tumors. Although the primary tumor may be resectable in about one half of the patients, this is almost invariably followed by the appearance of metastases (4). Various surgical series (5–7) have reported a median survival time of 2–3 years with surgery alone. For metastatic or locally recurrent disease, palliative treatments include surgery, radiotherapy, and mitotane. Except in selected cases none of these modalities of treatment has extended survival time significantly. Mitotane is currently the conventional treatment for patients whose tumor is not amenable to surgery. Initial reports about the activity of mitotane were quite encouraging (8). Subsequent reports have revealed that most of the patients are unresponsive to mitotane, and the responses are generally short-lived with no significant impact on the overall survival.

There are very little data in the literature on the activity of conventional or investigational chemotherapeutic agents except for occasional mention in broad phase I–II studies. Therefore, we have reviewed our experience with cytotoxic chemotherapy at the M. D. Anderson Hospital and Tumor Institute in the treatment of this tumor. The number of patients treated with cytotoxic agents is limited because of the rarity of this neoplasm and also because most of the patients were treated with mitotane alone.

MATERIALS AND METHODS

The charts of all the patients with a diagnosis of adrenal cortical carcinoma who received chemotherapy other than mitotane during the years 1960 through July 1979 were reviewed. In all patients, the diagnosis was confirmed by the M. D. Anderson Hospital pathologists. All of the patients had surgical excision of the primary tumor except two who had widely metastatic disease at the time of diagnosis. Mitotane was started after local recurrence or distant metastasis appeared. Cytotoxic chemotherapy was generally instituted after progression with mitotane.

Most patients had a metastatic workup including chest roentgenogram, liver scan, iv pyelogram,

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and, more recently, abdominal ultrasonogram. Cbc and blood chemistries were done routinely. A majority of the patients had basic endocrine evaluation including measurements of serum cortisol and urinary 17-hydroxy and -ketosteroids. Since there was no specific protocol, the choice of chemotherapeutic agent varied according to their availability and the choice of the treating physician. Responses were defined as follows: *complete remission (CR)*—the complete disappearance of all visible tumor; *partial remission (PR)*—a ≥ 50% objective regression of measurable tumor without increase in any of the previously involved areas or the appearance of new lesions; *minor regression (MR)*—an objective regression of > 25% but < 50% with no new disease or increase in any of the previously involved areas; *stable disease*—objectively and subjectively unchanged for at least 8 weeks; and *progression*—objective increase in measurable disease. Duration of PR was measured from the time the response was noted rather than from the start of treatment. Patients were considered to have received an adequate trial if they received two or more courses of treatment. All patients who received less than two courses or had a followup of < 2 weeks were considered to have had an inadequate trial. A Medline search was conducted for the preceding 13 years for all available literature on the chemotherapy of adrenal cortical carcinoma.

RESULTS

A total of 16 patients received cytotoxic chemotherapy, with 12 of them receiving an adequate trial of various drugs or drug combinations. Since some of the patients received more than one regimen at various times during their clinical course, a total of 27 trials, with 16 different regimens, were evaluable for response. Of these 27 trials, 17 utilized various single agents and ten used combinations. The patient characteristics are shown in table 1 and the results are summarized in table 2. Twenty of these 27 trials were without any therapeutic benefit. Seven of the 27 trials resulted in antitumor responses. Three patients achieved a PR, one achieved an MR, and three had stable disease. The PRs were of 3, 6, and 7 months' duration, respectively. The patient with an MR was lost to followup. Two patients had stable disease for 6 months each and one patient had stable disease for 2 months. The responses were observed in metastatic lesions in the liver, lungs, retroperitoneum, and subcutaneous tissue.

One patient with pulmonary metastases achieved a PR with a four-drug combination consisting of cyclophosphamide, vincristine, methyl-CCNU, and bleomycin (COMB). She was initially treated with mitotane and remained stable for 7 months. COMB chemotherapy was started when the lesions started progressing rapidly, and after three courses there was > 50% regression of a hilar mass, and significant regression of bilateral pulmonary nodules with the disappearance of some. A second patient with extensive disease in liver, retroperitoneum, and subcutaneous tissue achieved a PR with peptichemio. Prior therapy with mitotane and doxorubicin (Adriamycin) was ineffective. After six courses she had > 50% improvement in the liver scan, accompanied by some reduction in size of the subcutaneous nodules and abdominal disease. She received a total of 12 courses of peptichemio after which she developed progressive disease. A third patient with bilateral pleural effusions with hilar masses and enlarged liver was treated with doxorubicin given on a biweekly schedule. After 3 months of therapy she was totally asymptomatic and achieved a clinical CR with normalization of chest roentgenogram and liver scan. Three months later an exploratory laparotomy disclosed residual tumor around the contra-lateral adrenal gland. Doxorubicin was discontinued and radiation therapy was given; however, 8 weeks later she developed bilateral pulmonary metastases which did not respond to the re-institution of doxorubicin. One patient treated with vinblastine and melphalan showed an MR of bilateral pulmonary nodules. All of the patients who had stable disease had metastases in the lungs.

Four patients were unevaluable for response. One of them was felt to have questionable residual disease after surgery and began receiving 5-FU, cyclophosphamide, and actinomycin D. Treatment was discontinued after 11 courses and the patient is alive and well without any evidence of disease 4 years later. Another patient received actinomycin D and radiation therapy simultaneously and showed some regression of lung lesions. Two other patients received chemotherapy preterminally and died within 2 days of receiving it.

Since many different regimens were used, an attempt was made to analyze the activity of various broad categories of antitumor drugs. Alkylating agents including cyclophosphamide, melphalan, or peptichemio were used alone or in combination with other drugs in 12 patients, five of whom responded (two with PR, one with MR, and two with stable disease). Similarly, doxorubicin was used alone or in combination with other drugs in eight patients, three of whom showed a response (one with PR and two with stable disease); however, some of these were common to the previous group and had additionally received alkylating agents.

TABLE 1.—Patient characteristics

Patient No.	Age	Sex	Race	Disease site	Prior therapy	Cytotoxic chemotherapy*	Comments
1	59	F	W	Liver, retroperitoneum, skin	Surgery	Doxorubicin (2)† Peptichemio (12)‡ AMSA (2) Vinblastine (2) BCNU (2) Cisplatin (2)	PR with peptichemio therapy for 7 mos; no response to other drugs
2	52	F	W	Lungs	Surgery	COMB (6)	PR with COMB for 3 mos
3	26	F	W	Liver, retroperitoneum	Surgery, mitotane	Peptichemio (2)	Clinically manifest Cushing's syndrome
4	40	F	W	Lungs, liver	Surgery, mitotane	Peptichemio (2) Cyclophosphamide + doxorubicin (8) Cisplatin (5)	Stable disease with cyclophosphamide + doxorubicin for 6 mos, later with cisplatin for 2 mos
5	53	F	W	Lungs, liver	Surgery, mitotane	FAC (9) CMF (3) Anguidine (2)	Stable disease with FAC for 6 mos
6	11	F	W	Lungs, liver, pleura	Surgery	Doxorubicin (20)	PR for 6 mos
7	63	M	W	Lungs, liver, bone	None	5-FU + hydroxyurea (2)	
8	40	M	W	Liver, retroperitoneum	Surgery	Doxorubicin + streptozotocin (3)	
9	29	F	W	Lungs, bone, retroperitoneum	Surgery, mitotane	COMB (3) Doxorubicin (2)	Elevated cortisol and testosterone levels
10	19	F	W	Lungs, retroperitoneum	Surgery, mitotane	Vinblastine + melphalan (2)	MR, lung metastasis
11	28	F	W	Soft tissues, liver, lungs	Mitotane	5-FU + cyclophosphamide + CCNU	Clinically manifest Cushing's syndrome
12	27	F	W	Lungs, liver	Surgery, mitotane	Peptichemio (2) FAC (2) Methotrexate (3) AMSA (2) BCNU (2)	Clinically manifest Cushing's syndrome

*AMSA = 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; FAC = 5-FU, doxorubicin, and cyclophosphamide; and CMF = cyclophosphamide, methotrexate, and 5-FU.

†Nos. in parentheses = No. of courses of therapy.

‡Peptichemio is an analog of melphalan.

DISCUSSION

Adrenal cortical carcinoma is a highly malignant tumor with a very poor prognosis. Lipsett et al (9), reviewing the experience at the National Cancer Institute (Bethesda, MD) among 38 patients, found that 50% of the patients died within 2 years of onset of symptoms. Only 30% of the patients whose tumors were not amenable to curative surgery survived 2 years. Patients with local recurrence or metastatic disease are generally treated with mitotane. It was introduced by Bergenstal et al in 1960. Subsequently, Hutter and Kayhoe (8) published the results of its use among 138 patients. The study was conducted by the National Cancer Institute with the cooperation of 105 physicians across the country. Data were generated based on the reports of the treating physicians. An overall objective tumor regression rate of 34% was noted among 59 patients with measurable disease. However, no uniform criteria for antitumor response were applied. Any indication on the report forms of regression in measurable disease was accepted as evidence of response. The median duration of response to

TABLE 2.—Response to various chemotherapeutic regimens

Chemotherapy	No. of patients	No. of responses			Comments
		PR	MR	Stable	
COMB	2	1 (3 mos)*			Bilateral pulmonary metastasis regressed by 50%
FAC	2			1 (6 mos)	
CMF	1				
5-FU + CCNU + cyclophosphamide	1				
Doxorubicin + cyclophosphamide	1			1 (6 mos)	Stable pulmonary metastasis
Vinblastine + melphalan	1		1		
5-FU + hydroxyurea	1				
Doxorubicin + streptozotocin	1				
Doxorubicin	4	1 (6 mos)			*See* text for details of PR
Peptichemio	4	1 (7 mos)			Responded in liver, abdomen + soft tissues
BCNU	2				
Methotrexate	1				
Cisplatin	2			1 (2 mos)	Stable pulmonary metastasis
Vinblastine	1				
AMSA	2				
Anguidine	1				

*Nos. in parentheses = duration of response. Since some of the patients received > 1 regimen, the total No. of patients in this table are more than the group total of 12. Patients who did not have PR, MR, or stable disease progressed.

mitotane was 7 months. Subsequently, Hoffman and Mattox (10) reported the Mayo Clinic experience with mitotane. They reported tumor regression in four of 19 patients. The duration of response varied from 3 to 14 months. Hajjar et al (7) have reviewed the M. D. Anderson Hospital experience with mitotane, and noted one objective response among ten evaluable patients. They further commented that median survival in nine patients who were treated with surgery followed by mitotane was not significantly different compared to that of 12 patients who were treated with surgery alone.

Based on these reports it is obvious that the antitumor activity of mitotane against adrenal cortical carcinoma is marginal at best. Besides poor response rate, the gastrointestinal and CNS toxicity has been a major deterrent to its use.

Experience with cytotoxic chemotherapy against this tumor is very limited. Despite its significant impact in the treatment of various other malignancies, chemotherapy has been used infrequently in the treatment of this disorder, and only occasional reports of drug therapy besides mitotane are available. Lewinsky (11) while reviewing the clinical and pathologic features of adrenal cortical carcinoma in 178 cases noted that only two patients received cytotoxic drugs. One received methotrexate with no response and another received methotrexate, vincristine, and 5-FU in sequence, with a transient response. Antitumor responses have been reported in single patients with the nitrosourea, BCNU (12), cisplatin (13), and a combination of 5-FU and methotrexate (14). There is one reported cure with the use of 5-FU combined with mitotane in a patient who achieved a CR of disease in lungs, liver, and retroperitoneum after 4 months of treatment, and at autopsy 10 years later, there was no evidence of disease (15).

It is difficult to draw any firm conclusions regarding the activity of any particular drug or drug combination from the present series. Alkylating agents and doxorubicin appear to have some antitumor activity and deserve further study. In addition, 5-FU and nitrosoureas have been observed to induce tumor regression in isolated patients. As is the case in other rare tumors, multi-institutional

cooperative studies are needed to gather enough patients who could be treated with uniform protocols to detect active drugs and drug combinations.

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Enhancement of the Antitumor Activity of *Corynebacterium parvum* by Appropriate Adjustment of Dosage Schedules[1,2]

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SUMMARY

Single and multiple doses of *Corynebacterium parvum* ranging from 0.01 to 60 mg/kg as the total dose were tested at selected times against 10^6 P388 leukemia cells inoculated ip in BDF_1 mice. The minimum dose needed for maximum protection was determined according to the ability of the treatment to produce an increase in the lifespan (ILS) of mice. The ip dose required after transplant for significant ILS was 40 mg/kg as the total dose. The administration of a single dose 2 days prior to implant produced maximum antitumor effects at 1 mg/kg, with all other doses above and below this level being significantly less effective. Protection was achieved before implant, with the following maximum ILS recorded for each dose and time: 1 mg/kg on Day –2 = 110%; 60 mg/kg on Day –5 = 70%; 10 mg/kg on Day –2 = 50%; and 0.1 and 0.01 mg/kg on either Day –2 or Day –5 = 25%–30%. Cytotoxicity studies demonstrated that ^{51}Cr release after 1 mg/kg was more rapid in onset (peaking on Day 3 after treatment) than after 60 mg/kg (peaking on Day 5 after treatment). It was concluded that the proper adjustment of the dose and time of administration of *C. parvum* prior to tumor transplant enhanced antitumor activity of this immunopotentiator. Considerably higher doses were required after transplant for the desired effects to be achieved. The cytotoxicity studies demonstrated differences in tumor cell-killing which were dose-related and may be a contributing factor in the dose and time-related antitumor effects of *C. parvum*.

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Corynebacterium parvum has been shown to protect against various tumors (1–6), with the immune system involved in this process (7–10). This type of nonspecific protection by agents that are sometimes called immunopotentiators is of short duration and produces the best protection if the agent is placed in the same compartment as the target cells (11,12). One experimental approach which permits this close association involves the injection of both the immunopotentiator and the tumor cells into the peritoneal cavity. There is no assurance, however, that an immunopotentiator will produce antitumor activity (13). Evidence in the literature is also conflicting as to whether the dose of an immunopotentiator is a factor in the inhibition of experimental tumors (2,11,14).

While studies have demonstrated that *C. parvum* can induce nonspecific inhibition of a number of tumors in experimental animals, the importance of dose in relation to the time of treatment has not been adequately investigated. In some studies mul-

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tiple doses were tried in an effort to enhance effectiveness of *C. parvum* (2,5,8). In single-dose studies, Ikeda et al (3) and Mazurek et al (11) made limited changes in time and dose. With respect to the P388 tumor, Houchens et al (2) did vary the dose and time of administration of *C. parvum* and demonstrated an increase in the median survival time (MST) of mice receiving 10^6 P388 leukemic cells implanted by the same route.

These investigators suggested that the protective effect was independent of the dose administered and was the result of nonspecific stimulation of the host. This was apparent if *C. parvum* was given shortly before or after tumor cell inoculation in doses ranging from 9 mg/kg/injection for a single dose up to 70 mg/kg/injection for multiple-dose schedules. However, specific threshold doses for significant effects with *C. parvum* before as well as after transplantation of the P388 leukemia were not determined and the possible value of these low doses in achieving an antitumor response were not investigated. The present study, utilizing MST and in vitro cytotoxicity as endpoints, examined the importance of dose and time of treatment in selecting the minimum *C. parvum* treatment necessary to achieve maximum protection.

MATERIALS AND METHODS

Animals and tumor.—BDF_1 (C57BL/6 × DBA/2) male mice, 6–8 weeks old, were used in these studies. The P388 leukemia was supplied by Arthur D. Little, Inc, Cambridge, MA, and was maintained in DBA/2 mice with tests for antitumor activity being evaluated in BDF_1 mice by standard protocols described elsewhere (15). The tumor was implanted ip using 10^6 cells/mouse and the MST was determined in control and treated mice with antitumor activity expressed as the percent increase in lifespan (%ILS) as follows:

$$\frac{\text{MST treated-MST control}}{\text{MST control}} \times 100 = \%\text{ILS}.$$

The MST data (expressed as %ILS) were compared statistically by a median test for two samples utilizing the chi-square method. The assessment of possible differences in cytotoxic effects was made by the corrected t test for multiple comparisons. A $P < 0.05$ was considered significant.

Treatment.—*C. parvum*, as a suspension of Formalin-killed organisms (7 mg/ml), was supplied by Burroughs Wellcome Co, Research Triangle Park, NC. The suspension was either injected directly or diluted with 0.9% NaCl so that a constant volume of 0.2 ml was administered either ip, sc, or iv. The doses ranged from 0.01 to 60 mg/kg in either single or multiple doses at times indicated in each experiment.

Cytotoxicity assay.—The in vitro technique of Canty and Wünderlich (16) was employed to assess cytotoxicity. A total of 2×10^4 ^{51}Cr-labeled ascites P388 leukemic cells/micro-tissue culture plate were used as target cells. Peritoneal cells obtained by saline lavage from saline- and *C. parvum* (either 1 or 60 mg/kg ip)-treated BDF_1 mice not bearing a tumor were used as the effector cells for the control and treated groups, respectively. The effector:target cell ratio in both the treated and control groups was set at 40:1 in all experiments. The cells were suspended in minimum essential medium (GIBCO) supplemented with 10% fetal calf serum and incubated at 37°C for 4 hours in a humidified atmosphere of 95% air:5% CO_2. Cytotoxicity was calculated on the basis of ^{51}Cr release as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{treated } ^{51}\text{Cr release-control } ^{51}\text{Cr release}}{\text{control } ^{51}\text{Cr release}}.$$

RESULTS

Effect of Multiple Doses of *C. parvum* Administered After Tumor Transplant

When *C. parvum* was injected ip in doses ranging from 0.01 to 40 mg/kg on Days 1, 3, 5, and 7 after tumor transplant, antitumor activity was achieved only at the two higher doses (table 1). While the data suggested a dose-related relationship, a minimum dose of 10 mg/kg/injection was required to produce a significant effect.

Effect of Single Doses of *C. parvum* Administered Before Tumor Transplant

As shown in figure 1, single doses of 0.01, 0.1, 1, 10, and 60 mg/kg of *C. parvum* were administered

TABLE 1.—Effect of multiple doses of *C. parvum* administered after tumor transplant

Treatment and dose* (mg/kg/injection)	MST (days)	%ILS
Saline	10	—
C. parvum		
0.01	10	0
0.1	11.5	15
1	12	20
10	14.5	45
40	16	60

*Injections were made ip on Days 1, 3, 5, and 7 after tumor implant. Each treatment = determinations with 12 mice.

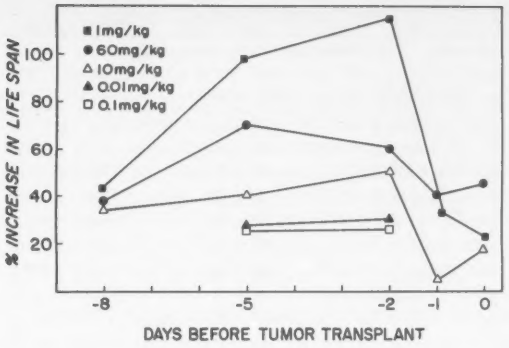

FIGURE 1.—Antitumor effects of single doses of *C. parvum* given ip at selected times before 10^6 tumor cells ip. Each point = %ILS determined with 12 mice.

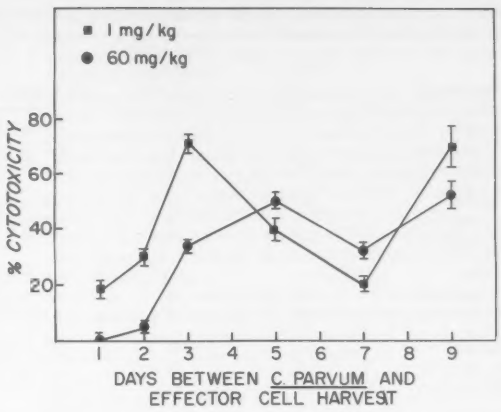

FIGURE 2.—% cytotoxicity against tumor cells by peritoneal cells after single ip doses of *C. parvum* given to non-tumor-bearing mice. Each point = mean ± SE determined from 5 mice.

ip as early as 8 days before and as late as the day of tumor cell inoculation. The maximum %ILS was observed using a single dose of 1 mg/kg injected on either Day –5 (95% ILS) or Day –2 (110% ILS) prior to tumor cell challenge. At all doses > and < 1 mg/kg, the protection was significantly less at both time periods. The 60-mg/kg dose produced a maximum effect on Day –5 (70% ILS), the 10-mg/kg dose produced a maximum effect on Day –2 (50% ILS), and the two lower doses, 0.01 and 0.1 mg/kg, failed to produce significant effects. As the time of treatment approached the time of tumor transplant, only the 60-mg/kg dose maintained a significant ILS above the control after Day –2. Doses of 60, 10, and 1 mg/kg administered 8 days before transplant produced essentially the same ILS (35%–40%) as shown in figure 1.

It was also found that a dose of 1 mg/kg sc or iv 2 days before transplant did not demonstrate any antitumor activity. Several multiple-dose regimens tested (not shown), ranging from 60 to 0.2 mg/kg/injection, offered no advantage over single doses when given prior to tumor cell challenge.

Cytotoxicity

The cytotoxicity assay results for two different single ip doses of *C. parvum* at 1 and 60 mg/kg are shown in figure 2. As can be seen, the cytotoxicity responses for the two doses were quite dissimilar. The 1-mg/kg dose produced a significant effect as early as the first day of treatment, with a maximum response achieved by Day 3. The 60-mg/kg dose, however, did not show an effect until Day 3, with a peak response achieved by Day 5. The peak cytotoxicity response observed on Day 5 with the 60-mg/kg dose was significantly less than the peak observed on Day 3 with the 1-mg/kg dose.

The yield of cells lavaged from the peritoneal cavity was increased more than threefold after *C. parvum* and was dose- and time-dependent (table 2). The ratio of lymphocytes:macrophages showed minimal changes throughout the study, while the yield of polymorphonuclear leukocytes (PMNs) peaked during the first day or two with either dose, and changes were minimal thereafter.

DISCUSSION

The present results are contrary to the claim by Houchens et al (2) that the anti-P388 tumor activity of ip *C. parvum* was unrelated to the dose administered. However, their study was largely restricted to treatment after tumor transplant, and critical doses prior to transplant with this immunopotentiator were not examined. What is particularly interesting about the present study was the finding that administration of a 1-mg/kg dose during a restricted time period before transplant (Day –2 and Day –5) produced a 95%–110% ILS which was significantly greater than that produced by the higher and lower doses tested (fig 1). Houchens et al (2) did not observe this effect when *C. parvum* was administered as a single dose 3 days before P388 tumor transplant because the lowest dose tested in that series was only 25.2 mg/kg. To produce a comparable ILS (> 90%) when treatment

TABLE 2.—No. of cells lavaged from the peritoneal cavity at various times after single doses of *C. parvum*

Treatment	Dose (mg/kg)	Time after treatment (days)	Total cells × 10^6*	Differential as % of total cells*		
				Lymphocytes	Macrophages	PMNs
Saline	—	1	2.5 ± 0.3	36 ± 8	64 ± 8	0
C. parvum	1	1	6.1 ± 1.0	39 ± 3	54 ± 3	7 ± 2
C. parvum	60	1	7.4 ± 0.7	29 ± 3	46 ± 4	25 ± 2
Saline	—	2	2.4 ± 0.4	31 ± 2	69 ± 2	0
C. parvum	1	2	4.9 ± 0.8	33 ± 3	57 ± 2	10 ± 3
C. parvum	60	2	8.5 ± 0.9	34 ± 4	47 ± 2	19 ± 4
Saline	—	3	3.0 ± 0.3	37 ± 2	61 ± 3	2 ± 1
C. parvum	1	3	6.6 ± 1.0	46 ± 3	54 ± 3	0
C. parvum	60	3	7.8 ± 0.9	36 ± 6	60 ± 5	4 ± 1
Saline	—	5	2.5 ± 0.4	43 ± 6	57 ± 6	0
C. parvum	1	5	3.9 ± 0.7	52 ± 3	46 ± 4	2 ± 1
C. parvum	60	5	7.0 ± 0.5	41 ± 5	54 ± 4	5 ± 1
Saline	—	7	3.4 ± 0.4	32 ± 3	68 ± 3	0
C. parvum	1	7	7.2 ± 0.3	53 ± 5	45 ± 5	2 ± 1
C. parvum	60	7	4.0 ± 0.8	47 ± 5	45 ± 5	8 ± 1
Saline	—	9	2.0 ± 0.4	50 ± 4	50 ± 4	0
C. parvum	1	9	5.9 ± 0.3	51 ± 2	47 ± 3	2 ± 1
C. parvum	60	9	3.4 ± 0.8	50 ± 3	49 ± 3	1 ± 1

*Each value = mean ± SE determined from 5 mice.

was instituted *after* transplant, a total dose of ≥ 100 mg/kg was required (2). This strongly suggests that not only is the response to *C. parvum* associated with dose and time of treatment in relation to tumor transplant, but it may also involve different dose-dependent mechanisms of tumor cell-killing. Bomford (17) related some of these effects of *C. parvum* with reduced resistance to tumor grafts. However, other studies have implicated more than one effector cell, including the macrophage, PMN, and so-called natural killer (NK) cell, in nonspecific killing (18–21). If true, this would contribute to the complexity of the response produced by a stimulus even when only dose and time factors are evaluated.

Since the MST with this tumor is directly related to the number of tumor cells injected (22), the more effective doses of *C. parvum* should induce the killing of larger numbers of tumor cells. Examination of the peritoneal cavity during the first few days after various treatments verified this conclusion since tumor cell numbers were reduced by effective treatments. It should be noted that the cytotoxicity with the 60-mg/kg dose of *C. parvum* (fig 2) is similar to that reported with a dose of 70 mg/kg in assays also employing peritoneal cells from BDF_1 mice and using the same method of assessment (2). The more rapid response achieved with the 1-mg/kg dose as compared to the higher dose (fig 2) could, in part, explain the greater antitumor effect of this dose when administered prior to tumor transplant.

The reasons for the significant differences in cytotoxicity seen during the first 3 days after treatment could involve suppression of effector cell activity (17) during the first few days after treatment with the 60-mg/kg dose and/or the involvement of more than one effector cell (18–21) in the early response with 1 mg/kg. The role of PMNs is probably minimal since they are largely absent by Day 3 after *C. parvum* (table 2) while there is still a significant difference in the cytotoxic response at this time period (fig 2). What is difficult to explain based on the cytotoxicity studies is why, if the 1-mg/kg dose produces a more rapid response to the killing of tumor cells, this dose is not consistently better than higher doses of *C. parvum* regardless of the time of treatment.

Several other points should be considered as a result of this investigation. *C. parvum* administered either iv or sc offered no protection, at least at the 1-mg/kg dose, while ip administration obviously did. This is in agreement with the studies of Mazurek et al (11) and Hibbs (12) in which it was stressed that the best protection against experimental tumors by nonspecific mechanisms is achieved when the stimulus and the target cells are placed in the same compartment at approximately the same time. Since macrophage-mediated cytotoxicity is usually observed after 12–15-hr incubation periods (23,24), the assessment of tumor cell-killing by ^{51}Cr release after 4-hr incubation suggests the

involvement of other cells, probably lymphocytes. In other studies now in progress with *C. parvum* and the ascites form of Sarcoma 180, similar dose-effect relationships were also found. Since NK cell activity following treatment with an immunopotentiator is apparently not influenced by specific immunization (25), and the tumor inhibition, with similar dose-response effects is not restricted to one tumor model, the possibility exists that NK cells are involved here. These cells have been implicated, with respect to the antitumor activity of *C. parvum*, in a number of other tumor models (20,21) in which cytotoxic effects were significant with the lowest dose tested (approximately 4 mg/kg ip). This, of course, does not rule out the reported involvement of macrophages in nonspecific tumor inhibition with *C. parvum* (19).

Finally, do the effects of time and dose described in this study with *C. parvum* and the P388 leukemia also apply to other experimental tumors as well as other immunopotentiators? As already indicated, our preliminary data suggest that similar results are possible with this agent and the ascites form of Sarcoma 180. Other studies in this laboratory involving BCG and glucan indicated that these agents were ineffective at high doses and that reduction of the dose and administration shortly before P388 transplant failed to produce antitumor activity. On the other hand, interferon, which has been suggested as the ultimate inducer of macrophage activation (26), does protect against the P388 tumor when administered in multiple doses after tumor implant (27). It would be interesting to see if interferon given prior to implant in low doses enhanced this protection.

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Fatal Pulmonary Bleomycin Toxicity in Cisplatin-Induced Acute Renal Failure[1]

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SUMMARY

A 53-year-old male being treated for a metastatic squamous cell carcinoma had fatal pulmonary toxicity as a result of bleomycin therapy. The toxic reaction was observed when the conventional dose regimen of bleomycin was given to the patient who had unrecognized cisplatin-induced acute renal failure. Bleomycin dosage should be reduced in patients with established acute renal failure and the combination of cisplatin and bleomycin should be carefully followed.

[Cancer Treat Rep 64:921–924, 1980]

Bleomycin and cisplatin are increasingly being used as combination chemotherapy in the treatment of certain resistant neoplasms. The major dose-limiting toxic effect of cisplatin is acute renal failure (1). Since the half-life of bleomycin is inversely related to creatinine clearance below values of 25–30 ml/min (2), prolonged bleomycin drug effects might be observed if drug dose is not reduced in patients with decreased renal function. The present report details fatal pulmonary bleomycin toxicity when a conventional dose regimen was given to a patient with unrecognized cisplatin-induced acute renal failure.

CASE REPORT

Patient 1 was a 53-year-old white male who was admitted to Portland Kaiser Hospital with a 5-cm, firm, right neck tumor proven on open biopsy to be metastatic squamous cell carcinoma. No primary site could be identified despite a complete workup including bronchoscopy, gastrointestinal endoscopy, and upper and lower gastrointestinal roentgenograms. On October 19, 1978, the patient received 250 mg of cisplatin (100 mg/m^2) iv together with 25 g of mannitol as an outpatient. Pretreatment renal function was normal with a BUN of 9 mg/100 ml, a creatinine of 1.0 mg/100 ml, and a creatinine clearance of 103 ml/min. On October 23, 1978, the patient reported 2 days of nausea and vomiting with concomitant decreases in urine volume. Fluids were encouraged, and on October 26, 1978, 35 units of bleomycin were given iv as a bolus and continued at a dose of 25 units daily until November 1, 1978. The total bleomycin dose was 185 units. On November 3, 1978, the patient was admitted to the hospital because of continued oliguria, nausea, vomiting, and generalized erythema of his skin. Laboratory data revealed a BUN of 114 mg/100 ml, a creatinine of 21.6 mg/100 ml, a calcium of 6.1 mg/100 ml, a phosphate of 6.2 mg/100 ml, a sodium of 127 mEq/liter, a potassium of 5.7 mEq/liter, and a hematocrit of 41%.

Because of continued oliguria and the need for dialysis, the patient was transferred to the University of Oregon Health Sciences Center on November 4, 1978. On admission, the patient was somnolent with a systolic blood pressure of 80. Oral mucous membranes were swollen and skin had profuse erythema and erythroderma. A 3 × 5-cm firm, fixed, right neck tumor was palpated. Bibasilar rales and rhonchi were audible and there was generalized edema. There were frequent myoclonic jerks and disconjugate gaze was observed. The wbc count was 4400/mm^3 with a 21% eosinophil count and the platelet count was 15,000/mm^3 with a 34% hematocrit level. Prothrombin and partial throm-

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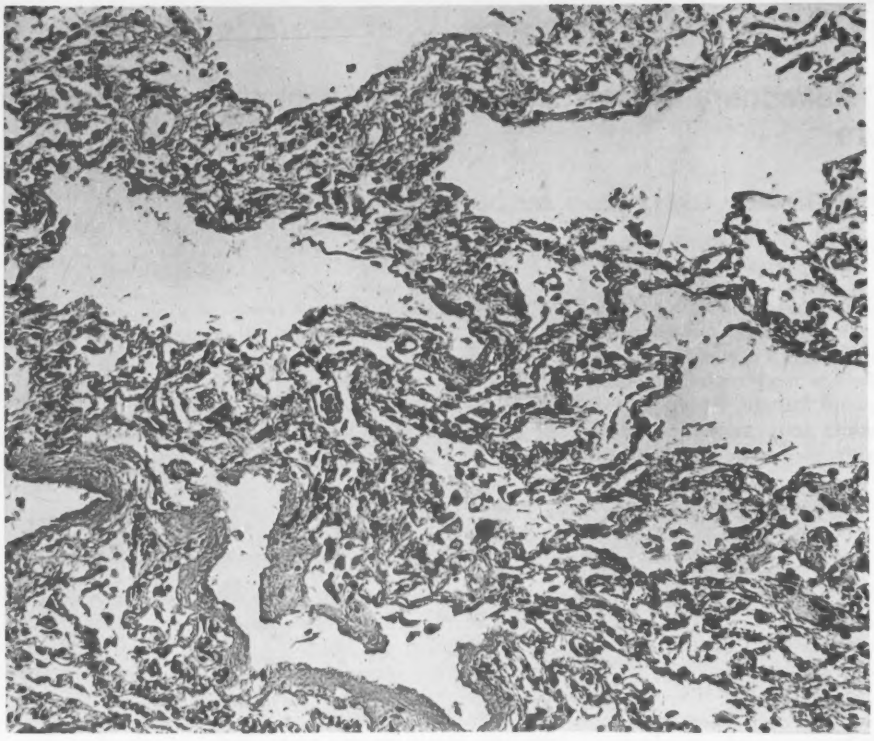

FIGURE 1.—Photomicrograph showing widening of alveolar and interlobular septa due to edema, mononuclear inflammatory cell infiltration, and fibrosis. Alveolar linings are denuded and covered focally by fibrinous exudate (hematoxylin and eosin stain, × 100).

boplastin times were markedly prolonged. Blood gases showed pO_2 68, pCO_2 28, and pH 7.49. Serum electrolytes showed a sodium of 128 mEq/liter, a potassium of 5.9 mEq/liter, a chloride of 98 mEq liter, and a bicarbonate of 20 mEq/liter; calcium was 7.7 mg/100 ml and phosphate was 4.5 mg/100 ml. BUN was 150 mg/100 ml and creatinine was 24 mg/100 ml. Liver function was normal except for the coagulation studies. Central venous pressure was 2 cm; H_2O and urine output was 5 ml/hr. Urine sediment showed 3+ protein without casts or cellular elements. Chest roentgenogram showed diffuse pulmonary infiltrates.

Treatment was begun with peritoneal dialysis, fluid therapy and dopamine with Swan-Ganz monitoring, platelet counts, fresh frozen plasma, and systemic steroids at a dose of 150 mg of prednisone daily. The patient died of progressive respiratory failure in 5 days despite intubation-assisted ventilation and positive-end expiratory pressure.

Necropsy revealed cutaneous erythema and epidermal exfoliation which were most severe on the anterior aspects of the chest, abdomen, and distal extremities. Mucosal ulceration and sloughing were extensive throughout the oral, nasal, and pharyngeal cavities, esophagus, trachea, and bronchi.

The lungs each weighed 1000 g. Though firm, the parenchyma was uniformly crepitant without evidence of focal, infiltrative disease. On section, the lungs were congested and septal markings were prominent. Microscopically, alveolar and interlobular septa were thickened by edema, small numbers of infiltrating mixed inflammatory cells, and delicate interstitial fibrosis (fig 1). Alveolar and bronchiolar lining cells were desquamating, and fibrinous membranes lined many air spaces. Segments of squamous metaplasia were all that remained of otherwise denuded bronchial and tracheal mucosa. Epithelial cells, particularly those of the metaplastic bronchial mucosa, demonstrated pronounced nu-

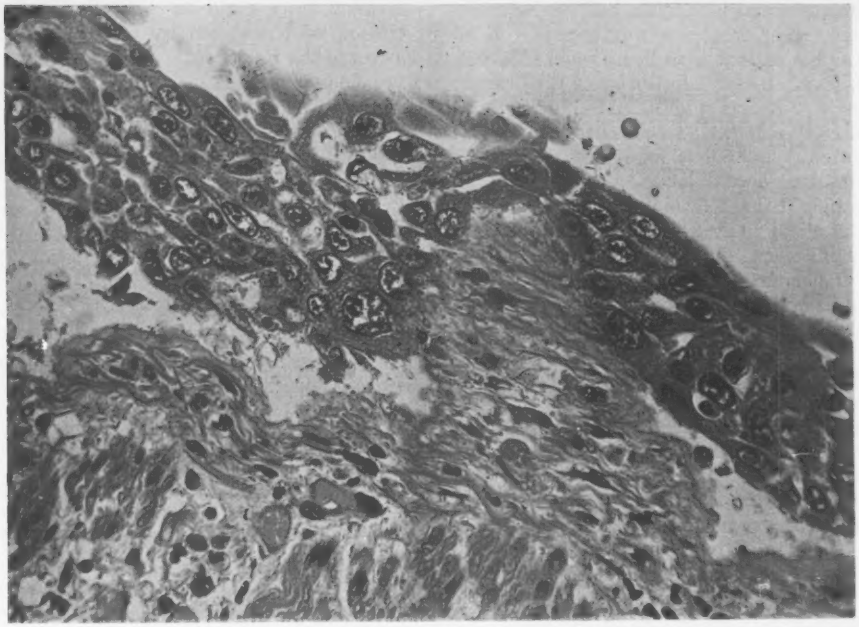

FIGURE 2.—Photomicrograph showing squamous metaplasia of the bronchial epithelium. Nuclear atypia is probably a cytotoxic drug effect (hematoxylin and eosin stain, × 250).

clear atypia (fig 2), changes which were probably an effect of bleomycin. No evidence of pulmonary infection was noted.

The kidneys weighed 230 and 250 g each, having normal extrarenal vessels, ureters, and pyelocaliceal structures. There were no parenchymal abnormalities evident on gross examination. Microscopic evidence of proximal tubular necrosis with active regeneration was noted.

Histologic examination of the 3-cm tumor of necrotic tissue and matted lymph nodes in the subcutaneous tissues of the right neck confirmed that it was metastatic keratinizing squamous cell carcinoma. No other site of tumor, either primary or metastatic, was found despite careful dissection of the structures of the head, neck, and lower respiratory tract.

DISCUSSION

Combination chemotherapy with cisplatin and bleomycin has been recently advocated in the management of advanced head and neck cancer (3). Although fatal toxic reactions have been reported with each drug separately, no increased adverse effects have been described for the combination of drugs. The major dose-limiting toxic reaction with cisplatin treatment is acute renal failure which occurs in as high as one third of the patients given doses similar to the reported case (> 95 mg/m²) (4). It is of interest that 14% of patients in two recent large series being treated with cisplatin and bleomycin had severe nephrotoxic reactions with 4% dying of acute renal failure (3,5). The mannitol administered with cisplatin in the present case did not prevent acute tubular necrosis. This is consistent with other data emphasizing the primary importance of hydration and extracellular fluid volume repletion rather than osmotic diuresis per se (1).

Bleomycin produces potentially lethal pulmonary interstitial pneumonitis and fibrosis in as high as 11% of treatment courses (6). Morphologic changes, as in our case, consist of diffuse alveolar damage progressing to interstitial pneumonia (7). This toxicity is dose-related with increased prevalence when the total dose is > 450 units (6). Bleomycin has a rapid plasma clearance with a terminal half-life of 1–1½ hours and a large renal excretion of unchanged drug (8). Although the total dose was only 185 units, our patient had oliguric acute renal failure with likely drug accumulation due to failure of renal excretion. Crooke et al (2) have demon-

strated that terminal bleomycin half-life increases exponentially when creatinine clearance is < 25 ml/min. In a single patient with frank renal failure no bleomycin was detectable in the blood 48–72 hours after a single iv injection of 30 units, although the half-life was extended to 21 hours (2). The present regimen of daily injections, however, would tend to maximize drug accumulation.

It is possible that the fatal pulmonary toxicity in this case was due to drug hypersensitivity as described by Holoye et al (9). Against this would be the lack of microscopic pulmonary inflammation, eosinophilia, and failure to respond to systemic steroids. Although the time course is relatively rapid for bleomycin-induced pneumonitis, it is possible that renal failure, per se, is a risk factor for this type of injury.

It is recommended that renal function be carefully monitored in patients receiving bleomycin and cisplatin combination therapy with downward adjustment of bleomycin dose if nephrotoxicity occurs. Ideally, chemotherapeutic agents with major renal excretion should be given prior to cisplatin. Careful attention must be directed toward pretherapy fluid and electrolyte repletion. In the absence of firm pharmacokinetic data in patients with marked renal insufficiency, bleomycin should be used no more frequently than every 48–72 hours. Alternatively, individual doses could be reduced. Finally, administration of bleomycin by constant infusion may increase the therapeutic to toxic ratio as compared to bolus therapy.

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Phase II Evaluation of the Combination of Triazinate, Cyclophosphamide, Doxorubicin, and *cis*-Diamminedichloroplatinum(II) in Patients With Advanced Adenocarcinoma of the Lung[1,2]

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SUMMARY

In an attempt to improve upon the 43%–48% regression rates noted for various CAP regimens consisting of cyclophosphamide, doxorubicin (Adriamycin), and *cis*-diamminedichloroplatinum(II) in various doses and schedules, triazinate was added to that three-drug combination, and the new combination (T-CAP) was evaluated in patients with advanced adenocarcinoma of the lung. T-CAP produced a regression rate of 57% with a 7-week increase in overall median time to progression and a 4-week increase in overall median survival compared to the best of the CAP schedules. More stomatitis and dermatitis were noted with the new combination, but myelosuppression was similar to that of the CAP regimens. These data suggest that further studies with triazinate should be conducted in patients with adenocarcinoma of the lung.

[Cancer Treat Rep 64:925–928, 1980]

In 1977, we reported on the activity of the combination of cyclophosphamide (CTX), doxorubicin (Adriamycin) (ADR), and *cis*-diammine-dichloroplatinum(II) (CDDP) (CAP-I) in non-small cell lung cancer (1). The regression rate noted for patients with adenocarcinoma of the lung (ACA) and no prior chemotherapy was 43% (six of 14 patients). We subsequently reported on another group of 19 patients treated with that cell type and identical chemotherapy in which the regression rate was 42% (2). In an attempt to increase the regression rate for CAP-I, we first tried adding VP-16-213 to the regimen after we had obtained a 12.5% (three of 24 patients) regression rate from VP-16-213 as a single agent (3). However, only a 35% (seven of 20 patients) regression rate was obtained (4), probably due to the fact that the myelosuppression from VP-16-213 prevented adding more than one third of the single-drug dose to the combination. We then tried increasing the dose of CDDP by 50% (CAP-II), but obtained only a modest increase in regression rate up to 45% (ten of 22 patients) (5).

Triazinate (TZT) is a triazine folate antagonist which, like methotrexate, inhibits dihydrofolate reductase. Rodriguez et al (6) had reported regressions in four of 31 patients (13%) with ACA. Unlike VP-16-213, the major toxic effects noted from TZT were stomatitis, diarrhea, and dermatitis, with myelosuppression noted in only 29% of the patients treated at a dose of 250 mg/m²/day for 3 days.

This study reports on the antitumor activity and toxicity noted from the combination of CAP and TZT (T-CAP) in patients with advanced ACA.

MATERIALS AND METHODS

Thirty-five patients were entered in the study after written informed consent was obtained. All patients had a diagnosis of ACA, the tumors being grade III or IV adenocarcinoma in 28 patients (80%) and grade I or II bronchoalveolar carcinoma in

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TABLE 1.—Pretreatment characteristics of 35 patients treated in the study

Characteristic	No. of patients
No. of patients	35
Ratio of males:females	22:13
ECOG PS	
0–1	21
2–3	14
Age in yrs	
Median: 57	
Range: 37–74	
Indicator disease	
Measurable	12
Evaluable	23
Disease sites*	
Lungs	32
Ipsilateral	19
Contralateral	4
Both	9
Bone	14
Brain	6
Liver	2
Other	9
Cell type	
Bronchoalveolar	7
Adenocarcinoma, grades III and IV	28

*Sites of disease exceed 35 because many patients had > 1 site of disease.

seven (20%). Tumor was identified outside the thorax in 33 patients and was widespread in the thorax in two. The majority of patients were males (63%) and had an Eastern Cooperative Oncology Group performance score (ECOG PS) of 0–1 (60%). All patients had measurable or evaluable tumor. The median age of the patients was 57 years (range, 32–74). No patient had received prior chemotherapy, five had had prior surgery, five had had prior thoracic irradiation, and four had had both prior surgery and thoracic irradiation. Thirty-two of the 35 patients had untreated or recurrent tumor in one or both lungs. Lung primary plus bone metastases was noted in 29% of patients and lung primary plus brain metastases was noted in 17%. This and other clinical data are shown in table 1. All patients with CNS metastases received CNS irradiation (3600 rads in 10 fractions; split course).

Indicator lesions were considered either measurable or evaluable. Measurable lesions required the presence of clearly defined tumor margins so that the longest perpendicular diameters could be measured. Evaluable lesions were visible and assessable, but not clearly measurable. A regression was a definite decrease in tumor size agreed upon by two observers. The response criteria are explained in great detail elsewhere (7). CNS progression alone did not constitute disease progression unless the CNS had been previously irradiated.

Patients were not eligible for study if any of the following conditions existed: a wbc count of $< 4100/mm^3$; a platelet count of $< 130{,}000/mm^3$; a serum creatinine ≥ 2.0 mg/dl; or significant active cardiac disease.

Chemotherapy was administered every 4 weeks initially, but could later be spread to every 5–6 weeks for better patient tolerance. TZT at a dose of 150 mg/m² was given as a 1-hour infusion in 500 ml of 5% dextrose in water, first on Day 1, after ADR on Day 2, and after CTX on Day 3. CDDP at a dose of 20 mg/m² was given as a 1-hour infusion in 500 ml of 5% dextrose in ½ normal saline (no mannitol or diuretics) on Days 1, 2, and 3 after TZT. ADR at a dose of 40 mg/m² was given by rapid iv infusion on Day 2 before TZT. CTX at a dose of 400 mg/m² was given as a rapid iv infusion on Day 3 before TZT (table 2). The drug scheduling was done in an attempt to lessen nausea and vomiting. It was our impression that CDDP was better tolerated as 1-hour infusions of 20 mg/m² for 3 days than CDDP given either as 40 mg/m² by rapid infusion (1) or 60 mg/m² as a 6-hour infusion (5). In addition, the ADR and CTX were given on 2 different days rather than both on the same day (1,5). At the start of treatment, the CDDP dose was decreased by 25% if the serum creatinine was 1.3 or 1.4 mg/100 ml or by 50% if the serum creatinine was 1.5 or 1.9 mg/100 ml. ADR and TZT doses were to be lowered by 25% or 50% if the total serum bilirubin was 1.3–2.0 mg/dl or > 2.0 mg/dl, respectively. Drug dosage adjustments were also made for interval toxicity.

RESULTS

All 35 patients were considered evaluable and all have been in the study for at least 6 months.

Regression Rates

Tumor regressions (complete response [CR], partial response [PR], and regression) were noted in 57% of patients (20 of 35) treated. Among the 23 patients with evaluable disease, regressions were noted in 48% (11 of 23 patients). Among the 12 patients with measurable disease, eight PRs and one CR were noted (CR plus PR = 75%). The regression rate for patients with an ECOG PS of 0–1 was 67% (14 of 21 patients) versus 43% (six of 14) for patients with an ECOG PS of 2–3. One patient with evaluable disease has been stable for

TABLE 2.—Routes, doses, schedules, and special instructions for chemotherapy

Agent	Route	Starting dose (mg/m²)	Days of administration	Special instructions
TZT	Iv, 1-hr infusion	150	1, 2, 3	Dilute in 500 ml of 5% dextrose in water; 1st on Day 1; after ADR on Day 2; after CTX on Day 3
CDDP	Iv, 1-hr infusion	20	1, 2, 3	Dilute in 400 ml of 5% dextrose in ½ normal saline; given after TZT on Days 1, 2, 3
ADR	Iv, 5-min infusion	40	3	Given before TZT on Day 2
CTX	Iv, 5-min infusion	400	3	Given before TZT on Day 3

> 9 months and will be included with the regressors in subsequent discussions on time to progression, regression duration, and survival. Of the 14 patients with disease progression, six had progression after one course, four after two courses, and one each after three, four, five, or six courses of T-CAP.

Time to Progression and Survival

Time to disease progression and survival from the first day of treatment were estimated from Kaplan-Meier curves and analyzed for differences using Gehan-Wilcoxon two-sided test statistics. Median time to disease progression, calculated from Day 1 of treatment, was 21 weeks for all patients and ranged from 8 weeks for patients having no tumor regression to 36 weeks for patients experiencing a tumor regression (fig 1). For the 20 patients with eventual tumor regression, the first notation of regression occurred after the first treatment in 45% (nine of 20 patients), after the second treatment in 35% (seven of 20), and after three treatments in the remaining 20% (four of 20). The difference in time to progression between regressors and nonregressors was significant ($P < 0.0001$). The median survival for all patients was 29 weeks and ranged from 14 weeks for patients experiencing no tumor regression to > 1 year (fig 2) for patients experiencing a tumor regression ($P = 0.0004$).

Toxic Effects

Nausea and vomiting occurred in all patients and was generally moderate to severe on Day 1 and none to moderate on Days 2 and 3. All patients had alopecia.

Stomatitis, probably from the combination of TZT and ADR, was noted in 17 of 35 patients (49%) (mild in five, moderate in ten, and severe in two). TZT dermatitis was noted in 11 of 35 patients (31%) (mild in six, moderate in three, and severe to the point of exfoliation in two).

Clinically apparent ototoxicity from CDDP was noted in one patient. No symptomatic CDDP neurotoxicity was observed. Total cumulative doses of CDDP ranged from 60 to 486 mg/m².

All patients had normal serum creatinine values (≤ 1.2 mg/dl) at the start of treatment. Serum creatinines increased to > 1.2 mg/dl in ten patients (29%) and ranged from 1.35 to 2.4 mg/dl (only one > 1.6 mg/dl). All elevated creatinine values decreased or remained stable in patients treated again with CDDP at a reduced dose.

Interval blood cell counts following the first

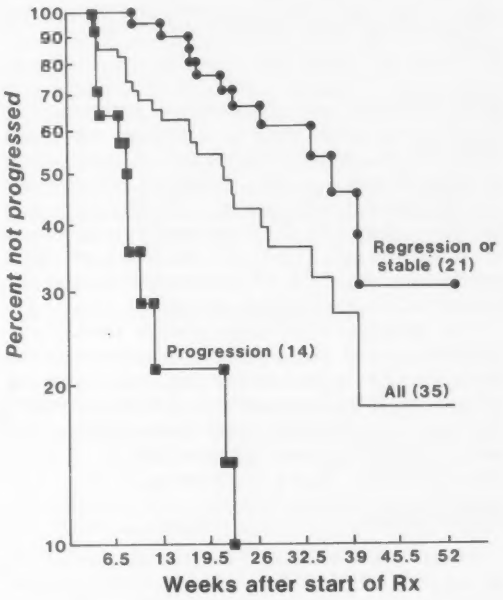

FIGURE 1.—Time to disease progression calculated from the start of chemotherapy. Among the 21 patients noted as having "regression or stable disease," 20 had tumor regression and only 1 was stable (> 9 mos).

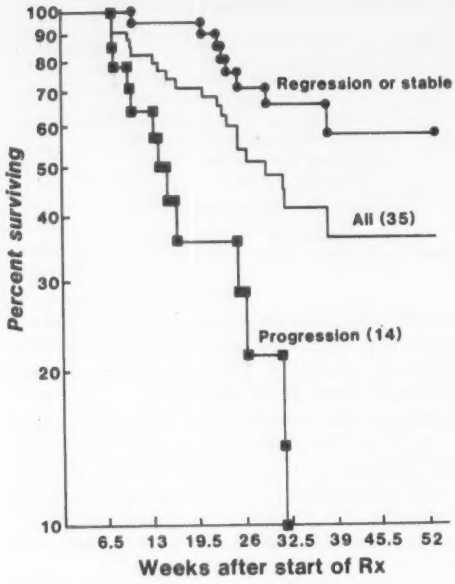

FIGURE 2.—Survival calculated from the start of chemotherapy. Among the 21 patients noted as having "regression or stable disease," 20 had tumor regression and only 1 was stable (> 9 mos).

course of T-CAP were obtained in 31 patients. Leukopenia (wbc count nadir < 4100/mm³) was noted in 65% of those 31 patients (median wbc count nadir of 2400/mm³; range, 1000–3900/mm³). Thrombocytopenia (platelet count nadir < 130,000/mm³) after the first course of T-CAP occurred in only four of 31 patients (range, 67,000–127,000 cells/mm³). With multiple courses of T-CAP, decreases in hemoglobin values to 1.5–3.0 g/dl were common.

Two episodes of probable allergic reactions to TZT were noted. One consisted of urticaria developing over the vein in which the drug was being infused and the other consisted of fever and chills. TZT was discontinued in both those patients with subsequent therapy consisting of CAP.

DISCUSSION

T-CAP was evaluated for antitumor activity in 35 patients with advanced ACA. T-CAP produced a 57% regression rate and an overall median survival of 29 weeks. Patients with tumor regressions experienced a significantly prolonged time to disease progression (36 versus 8 weeks) and significantly prolonged survival (> 52 versus 14 weeks) compared to patients experiencing no tumor regression.

TZT was added to the combination at 60% of the single-drug full dose. That full dose was associated with a 13% regression rate in patients with ACA. The regression rate of 57% with T-CAP was approximately 15% greater than for CAP-I with CDDP at a dose of 40 mg/m² and approximately 10% greater than for CAP-II with CDDP at a dose of 60 mg/m².

Compared to the best previous regimen (CAP-II with CDDP, 60 mg/m²), T-CAP increased median time to disease progression from 14 to 20 weeks for all patients and from 28 to 36 weeks for regressors. In addition, it added 4 weeks to the overall median survival (from 25 to 29 weeks) despite the fact that there were more T-CAP patients with ECOG PSs of 2–3 (40% versus 32%).

TZT added both dermatitis and stomatitis to side effects of the CAP regimen. However, both of these toxic effects were well-controlled by a reduction in the TZT dose (30% for moderate degree; 50% for severe degree). TZT did not appear to increase the degree and frequency of myelosuppression compared with CAP at either dose of CDDP (4,5).

The above data suggest, but do not prove, that TZT, used in combination with CAP, has a role in advanced ACA. Proof of this could be obtained only through a randomized trial of CAP versus T-CAP.

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Hepatotoxicity of Hycanthone in Patients With Metastatic Breast Cancer[1,2]

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SUMMARY

Hycanthone is an antischistosomal drug with promising antitumor activity against experimental animal tumors. In phase I clinical trials, hepatitis was the dose-limiting toxic effect and a dose of 60–70 mg/m²/day for 5 days was reported as the maximum-tolerated dose. In a phase II study of hycanthone in patients with breast cancer we have recently observed severe hepatotoxicity, even at lower doses, which resulted in two drug-related deaths. From this study and the previously available data, we conclude that this drug is too toxic for human trials at the currently recommended doses. Its radiosensitizing effect may be worth exploring at a lower dose level.

[Cancer Treat Rep 64:929–932, 1980]

Hycanthone is a hydroxymethyl analog of lucanthone which is a known antischistosomal agent (1). It is structurally related to actinomycin D and has a similar biochemical mode of action in being a DNA intercalator (2). Hycanthone has significant antitumor activity in experimental tumors such as L1210 and P388 leukemias and Walker 256 carcinosarcoma (3,4). It has been found to inhibit DNA and RNA synthesis without affecting protein synthesis in phytohemagglutinin-stimulated lymphocytes (4). Based on these data hycanthone has been tested in phase I clinical trials (5,6). The maximum-tolerated dose of hycanthone from phase I studies was 60–70 mg/m²/day × 5 days. Hepatitis was noted to be the major dose-limiting toxic effect. No significant antitumor response was observed in phase I studies. However, phase II studies are warranted to fully explore the potential antitumor activity of this agent, especially since it has no hematologic toxicity. We initiated a phase II study of hycanthone for treatment of patients with metastatic breast cancer resistant to standard drugs. Unfortunately, we encountered an unexpected degree of hepatic toxicity, and the study had to be terminated when two patients died from drug-related hepatic injury.

MATERIALS AND METHODS

Seven patients with advanced refactory breast cancer were entered in this phase II protocol. All patients had progressive disease and had previously received treatment with standard chemotherapy and hormonal agents. The starting dose of hycanthone was 70 mg/m²/day × 5 days. All patients had pretreatment liver function studies (serum bilirubin, SGOT, and SGPT) which were repeated before the fourth dose, and twice weekly thereafter. If SGOT, SGPT, and/or bilirubin were elevated to more than four times the normal value, the remainder of the hycanthone course was suspended and subsequent courses were administered at 50–60 mg/m²/day × 5 days. Treatment courses were administered at 3-week intervals if disease had not

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progressed and liver functions had completely recovered. Informed consent was obtained from all patients according to institutional policy.

RESULTS

All seven patients who received hycanthone during this abortive phase II trial (table 1) had had extensive prior chemotherapy. All patients were white females whose ages ranged between 43 and 64 years. Six of the seven patients had normal liver functions before initiation of hycanthone. One patient with metastatic disease in the liver had an elevated bilirubin of 1.4 mg/dl and an SGOT of 150 IU before treatment was initiated. Six of these seven patients manifested hepatotoxic effects, with two of them having severe hepatic damage resulting in death. One of the latter two patients was a 43-year-old woman with metastases in bones and liver. She had normal SGOT and bilirubin on the day treatment began. The liver scan was positive but stable prior to hycanthone therapy. She started receiving hycanthone at a dose of 70 mg/m²/day × 5 days. After 3 days of treatment her SGOT increased to 391 IU, her bilirubin rose to 3.7 mg/dl, and her prothrombin time was > 100 seconds; hycanthone was discontinued. The next day her SGOT increased to 1870 IU and her clinical status deteriorated rapidly; she died on the fifth day. Unfortunately, permission for autopsy was not granted. A second patient with metastatic disease in the lungs, bone, and brain started receiving hycanthone at a dose of 70 mg/m²/day × 5 days. She received a full 5-day course since after 3 days of treatment the SGOT was elevated only mildly (51 IU). She manifested a delayed rise in SGOT and bilirubin on Day 7 (82 IU and 1.4 mg/dl, respectively) which returned to normal by Day 10. Three weeks later a second course was started at a dose of 70 mg/m²/day × 5. It was suspended after 3 days because of elevation of SGOT to four times the normal value. A third course was started at a dose of 70 mg/m²/day × 5 days. On Day 4 her SGOT was three times the normal value and her bilirubin was 1 mg/dl; hycanthone was continued for the full 5 days. On Day 8 she was hospitalized with a bilirubin of 4 mg/dl and an SGOT of 318 IU. Her liver function tests deteriorated progressively, with the bilirubin reaching a peak value of 33 mg/dl on Day 24. The SGOT peaked at 318 IU on Day 8 and subsequently fell and remained low. SGPT reached a peak of 238 IU on Day 8 and subsequently fell and remained around 40 IU. The patient developed severe clotting abnormalities. A liver scan was negative. She eventually died of disseminated intravascular coagulation and hepatic failure. The bilirubin on the day of death was 32 mg/dl, with a direct bilirubin of 11 mg/dl.

Of the remaining four patients who manifested hepatotoxic effects, two received three courses each, one received five, and the other received one. Two patients had a starting dose of 70 mg/m²/day × 5 days, one had a starting dose of 50 mg/m²/day × 5 days, and a third had a starting dose of 60 mg/m²/day × 5 days. Only one of these patients was able to receive the full 5 days of treatment. No patients had liver metastases. They manifested a twofold to tenfold increase in SGOT with the bilirubin not exceeding 1.5 mg/dl. The peak rise in enzymes was noted on Days 4–5 with practically complete recovery by Day 10 in all the patients.

One patient received a full dose of 70 mg/m²/day × 5 days without significant hepatic toxicity. This patient had received no prior exposure to methotrexate, a known hepatotoxin. Her SGOT increased to twice the baseline value on Day 3 but subsequently decreased.

Of a total of 17 courses of treatment administered, only four courses reached a total dose of ≥ 300 mg/m², which equals the dose recommended by the Mayo Clinic report (6) for phase II studies. Only one of these courses had no significant toxicity while the other three had toxicity with one death. Twelve of 13 courses using a total dose of between 100 and 250 mg/m² also resulted in varying degrees of hepatic toxicity, predominantly manifested as elevated SGOT (30–1870 IU), with one death. Only one course at a total dose of 250 mg/m² resulted in no significant toxicity. Thus, only two of 17 courses showed no significant elevation of SGOT. No hematologic toxicity or antitumor response was seen.

DISCUSSION

Hycanthone is a widely used antischistosomal drug with minimal morbidity when used as a single dose of 3 mg/kg or 120 mg/m². Acute hepatitis has been recorded occasionally with its use (1,5–8). Farid et al (7) reported two cases of acute hepatic cell necrosis in children treated with hycanthone for schistosomiasis.

Kallmeyer et al (8) observed two cases of hepatorenal failure following repeated therapy with hycanthone. Both of these patients also received parenteral phenothiazine to control vomiting. In addition, one of these patients also had a past history of infectious hepatitis, and 1 week before the administration of hycanthone, was taking a high-dose estrogen preparation for contraception. Kallmeyer et al (8) speculated that previous hepatic injury or phenothiazines possibly potentiated the hepato-

TABLE 1.—Clinical summary of patients treated with hycanthone

Patient No.	Age (yrs)	Sites of metastases	Prior chemotherapy*	Hycanthone course No.	Hycanthone dose in mg/m² (total)	Values for SGOT in mU/bilirubin in mg/dl at— Pretreatment	Day 4	Peak (Day)	Recovery (Day)	Comments
1	43	Liver, bone	FAC-CMF, tamoxifen, anguidine	1	70 × 3 (210)	36/0.9	1870/5.8	1870(4)/5.8(4)		Died on Day 5; liver not palpable; also had seizures on Day 4
2	45	Liver, spleen, bone marrow	FAC-CMF, anguidine, tamoxifen, megace	1	60 × 5 (300)	150/1.4	184†/1.4	184†(4)/1.4(4)	135/1.4	Died of progressive disease; autopsy performed; no evidence of liver damage; extensive tumor in liver
3	55	Brain, lungs	Ftorafur, doxorubicin, dibromodulcitol	1	70 × 5 (350)	27/0.3	44/0.4	44(4)/0.4(4)	35/0.3	
4	55	Soft tissue	FAC-CMF, high-dose methotrexate, mitomycin C, hexamethylmelamine, vincristine	1	70 × 3 (210)	23†/0.5	74†/0.7	74†(4)/0.7(4)		Progressive disease; hycanthone discontinued
				2	70 × 3 (210)	10†/0.3	50†/0.6	50†(4)/0.6	15†(8)/0.6(8)	
				3	50 × 5 (250)	29†/0.5	30†/0.5	30†(4)/0.6(6)	17†(7)/0.7(7)	
5	43	Bone, soft tissue	FAC, LAM, HOM, tamoxifen	1	70 × 3 (210)	47/0.4	500†/1.5	500†(4)/1.5(5)	37(15)/0.4(15)	Progressive disease; hycanthone discontinued
				2	60 × 2 (120)	37/0.4	94/0.7	94(4)/0.7(4)	29(5)/0.5(5)	
				3	60 × 3 (180)	35/0.4	169†/0.8	169(4)/0.8(4)	30†(9)/0.6(9)	
6	64	Bone marrow	FAC, CMF, tamoxifen	1	50 × 2 (100)	39/0.6	41†/0.6	41†(4)/0.6(4)		Progressive disease
				2	50 × 3 (150)	41/0.6	97/0.6	97(4)/0.6(4)	28†(8)/0.6	
				3	50 × 2 (100)	21/1.0	123†/1.0	123†/1.0(4)		
				4	50 × 3 (150)	56/0.8	70†/1.1	70†/1.1(4)	31†(25)/0.8(25)	
				5	50 × 2 (100)	36/1.1	117†/1.4	117†(4)/1.4(4)	41(8)/1.2(8)	
7	53	Lungs, brain	VAC-FUM, CMF, peptichemio	1	70 × 5 (350)	31/0.5	51/0.4	104†(8)/1.4(8)	14(14)/0.6(20)	Vomiting + phlebitis
				2	70 × 3 (210)	27/0.4	82†/1.2	82†(4)/1.2(4)	23(11)/0.5(17)	
				3	70 × 5 (350)	23/0.5	62†/1.0	318†(11)/33(24)		Died with elevated bilirubin, severe bleeding

*FAC = 5-FU + doxorubicin + cyclophosphamide; CMF = cyclophosphamide + methotrexate + 5-FU; HOM = hexamethylmelamine + vincristine + mitomycin C; LAM = L-asparaginase + methotrexate; VAC = vincristine + doxorubicin + cyclophosphamide; FUM = 5-FU + methotrexate.
†SGOT was obtained by single channel (range, 5–20 mU) while other values were obtained from SMA (range, 7–40 mU).

toxicity of hycanthone. Dennis and Kobus (1), in an extensive review of the literature, found 40 cases of adverse reactions with hycanthone use. Seventeen of these were fatal, with autopsy revealing hepatic necrosis. Most of these patients had preexisting liver disease due to schistosomiasis, but a precipitating or contributory role of hycanthone could not be excluded. However, the authors felt that all but two or three of these deaths could have been prevented if hycanthone had been used with due precautions.

Hepatotoxicity in the form of elevated SGOT and bilirubin has been observed in monkeys and dogs exposed to hycanthone. Legha et al (5), in their phase I study from the M. D. Anderson Hospital and Tumor Institute, noted hepatitis as the major complication, with one drug-related death (5). Kovach et al (6), in a phase I study from Mayo Clinic, reported that two patients developed severe hepatic injury following the administration of hycanthone; however, both of them recovered from the hepatotoxicity.

Generally, the hepatotoxicity was manifested in the form of elevated SGOT and SGPT noted as early as Day 4 after the beginning of treatment and was occasionally followed by elevation of bilirubin a few days later. Alkaline phosphatase was rarely elevated and the liver was usually not enlarged. The Mayo Clinic study noted some elevation of SGOT at all doses used (range, 30–100 mg/m²/day × 5) (6). In the present study 15 of the 17 courses produced some degree of elevation of SGOT with two patients dying from the hepatic injury.

Histologic studies done in patients who developed toxic reactions revealed a pattern of acute toxic hepatitis, which was not consistent with viral hepatitis or hypersensitivity to drugs (7). Histologic studies in animals who manifested hepatotoxic effects showed hepatic congestion and hemorrhages without any necrosis. Kovach et al (6) did liver biopsies on two patients; one patient showed nonspecific changes in parenchyma and the second patient, who developed severe toxicity, showed central lobular necrosis. Unfortunately, the histologic nature of hepatic injury in our patients could not be ascertained since none of them had biopsy or autopsy.

There has been some speculation regarding the role of pre-existing liver disease or concomitant use of potentially hepatotoxic drugs with hycanthone aggravating the hepatic injury. None of the patients in the present study received phenothiazines; however, all except one patient had previously received methotrexate, a known hepatotoxin, and this could have accounted for the increased incidence of hepatotoxicity in this study compared to previous experience.

Even though none of our patients had histologic proof of hepatic necrosis, it was clear on clinical grounds that they had acute toxic hepatitis. Because of the high incidence and severity of hepatotoxicity of hycanthone with a dose range lacking antitumor activity, we feel that this drug is too toxic for evaluation as an antitumor agent. Based on its usefulness as a radiosensitizing agent, lower doses of hycanthone could be further explored for that purpose (9).

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Ifosfamide, Methotrexate, and Vincristine (IMV) Combination Chemotherapy as Secondary Treatment for Patients With Malignant Lymphoma[1,2]

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The treatment of patients with disseminated lymphoma of aggressive histology ("unfavorable" types) who fail to achieve a complete remission with primary chemotherapy has uniformly been associated with poor results (1–3). Patients relapsing from a remission induced by a doxorubicin (Adriamycin)-containing regimen generally respond poorly to salvage regimens. Even though second remissions can be obtained, these are usually only partial in nature and short-lasting (4,5). Most of the time, these patients are treated with phase I or II drugs due to the generally poor results achieved with conventional agents. On the other hand, those patients with indolent histologic types ("favorable" histologies) can achieve a relatively high response rate after having failed initial chemotherapy. However, the response rate and the duration of response also progressively decrease with time.

Ifosfamide, an analog of cyclophosphamide, is effective in an animal model resistant to cyclophosphamide (6). We recently published our experience with ifosfamide as single-agent therapy for patients with lymphoproliferative disorders refractory to front-line chemotherapy regimens (5). A partial response rate of 47% obtained in these patients encouraged us to investigate this drug further in combination with methotrexate and vincristine. This drug is primarily eliminated in the urine (6). In order to reduce the risk of bladder toxicity, the dose was fractionated over a 5-day period using the method described by Rodriguez et al (7) in the past. The selection of the other two drugs was based on a recent study by Lauria et al (8) in which a kinetically designed regimen utilizing cyclophosphamide, methotrexate, and vincristine resulted in a high response rate in previously untreated patients. This study reports on the results with the combination of ifosfamide, methotrexate, and vincristine (IMV) in 30 patients with malignant lymphoma resistant to conventional chemotherapy regimens. This combination may have potential value in the management of advanced malignant lymphomas.

MATERIALS AND METHODS

Patients with malignant lymphoma, other than Hodgkin's disease, were considered candidates for therapy with the IMV regimen if they had failed to respond to or relapsed on combination-chemotherapy regimens that included cyclophosphamide and doxorubicin. The IMV regimen included ifosfamide at a dose of 1 g/m^2 iv in 1000 ml of dextrose in water over 2 hours daily × 5, methotrexate at a dose of 30 mg/m^2 im or iv on Days 3 and 14, and vincristine at a dose of 2 mg iv on Days 1 and 14. The courses were repeated every 21 days if the patient had recovered from toxicity. In order to prevent bladder toxicity from ifosfamide, patients were encouraged to increase their fluid intake for the 5 days they were receiving this drug. They were also instructed to urinate as frequently as possible, preferably every hour, in order to reduce the exposure time of the bladder to the metabolites of ifosfamide. If gross hematuria appeared, ifosfamide was deleted from the regimen. If moderate to severe neuropathy was present, vincristine was omitted until symptoms improved.

Cbcs with differential and platelet counts were obtained twice a week while the patients were receiving therapy. Urinalyses were obtained on the first 5 days of treatment when the patient was receiving ifosfamide. If significant mucositis was present on Day 14 of treatment or if the absolute granulocyte count was < 500/mm^3 or the platelet count was < 75,000/mm^3, the second dose of meth-

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otrexate was omitted. If the lowest absolute granulocyte count from the previous course was < 500/mm³ or if the platelet count was < 75,000/mm³, the dose of ifosfamide and methotrexate during the subsequent course was reduced by 25%. If morbidity such as infection or hemorrhage was associated with any degree of myelosuppression during a prior course, the subsequent course was also reduced by 25%. The dose of ifosfamide and methotrexate was increased by 25% if the lowest absolute granulocyte count was > 1000/mm³ and if the platelet count was > 150,000/mm³.

A complete remission (CR) was defined as the disappearance of all evidence of disease and symptoms related to the disease for a minimum of 2 months. At least two courses of treatment were delivered before assessment of response was carried out. In order to evaluate response, all baseline examinations considered abnormal (including biopsies) were repeated. Maintenance therapy was continued until relapse occurred or up to 1 year after the point when CR was achieved. A partial remission (PR) was defined as a > 50% reduction in the sum of the product of the diameters of measured lesions lasting for at least 1 month. No simultaneous increase in the size of any lesion or the appearance of new lesions could occur. Response less than PR was defined as a < 50% regression of the tumor. Early deaths were considered as failures and these patients were not excluded from analysis. The duration of survival was measured from the time the patients started therapy with the IMV regimen. The method of Kaplan and Meier (9) was used to plot survival curves. The generalized Wilcoxon test was utilized to test differences in survival (10).

For purposes of this analysis, diffuse histiocytic (DHL), diffuse poorly differentiated lymphocytic (DPDL), diffuse undifferentiated (DUL), and nodular histiocytic (NHL) lymphomas were considered as aggressive histologic types. The nodular poorly differentiated lymphocytic (NPDL) and diffuse well-differentiated lymphocytic (DWDLL) lymphomas were considered indolent types.

Ifosfamide was supplied by the Mead Johnson Research Center (Evansville, IL). Informed signed consent was obtained from all patients according to institutional policy.

RESULTS

Thirty patients were entered in this study. Patient characteristics are summarized in table 1. All patients had previously received doxorubicin and cyclophosphamide. Most had received chemotherapy with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (1) and had advanced (stage IV) disease at the start of IMV therapy. The median number of prior courses of chemotherapy delivered was ten (range, two to 85 courses) (table 1). For the purpose of analysis, the response of patients with aggressive ("unfavorable") histologic types is evaluated separately from that of patients with indolent ("favorable") types. The overall response rate (CR + PR) was 46% for the 22 patients with aggressive histologic types (table 2). The most frequent histologic subtype in this group, DHL, showed an overall response rate of 42%. The CR rate for DHL was 18% (three responses among 17 patients), and two of these patients are still in CR at 28+ and 33+ months (fig 1). Chemotherapy has been discontinued in both of these patients. A significant advantage in terms of survival was found for patients who achieved a CR or PR as compared to those who failed to respond or who only had a minimal response (less than PR) (fig 1).

Only eight patients with indolent histologic types have been entered in this study. The overall response rate for these types was 50% (two CRs and two PRs). The two patients with CR lasted 15+ and 18 months while the two patients with PR lasted 8 and 14 months. None of the four patients

TABLE 1.—Characteristics of patients receiving IMV

Histologic type	No. of patients	Stage IV/III	Median No. of prior chemotherapy courses (range)
Aggressive			
DHL	17	16/1	9 (2–34)
DPDL	2	2/—	5,5
DUL	2	2/1	2,6
NHL	1	—/1	85
Indolent			
NPDL	7	7/—	18 (6–36)
DWDLL	1	1/—	17
Total	30	28/2	10 (2–85)

TABLE 2.—Response of aggressive histologic types ("unfavorable") to IMV

Type	No. of patients	No. of responses (%)*				
		CR	PR	PR	Inadequate trials	Failed
DHL	17	3 (18)	4 (24)	3 (18)	2 (12)	5 (29)
DPDL	2	—	2 (100)	—	—	—
DUL	2				1 (50)	1 (50)
NHL	1		1 (100)			
Total	22	3 (14)	7 (32)	3 (14)	3 (14)	6 (27)

*Overall (CR + PR) response excluding inadequate trials = 53%. Overall (CR + PR) response including inadequate trials = 46%.

who responded to therapy have died, while two of the four who failed to respond have already died. Due to the small numbers involved, statistical analysis of survival in these patients is not pertinent.

Table 3 shows the response to IMV as related to the prior response to primary chemotherapy regimens. There was no significant difference in the response to IMV between patients who achieved a CR or a PR with front-line chemotherapy. However, patients who showed refractoriness to primary chemotherapy were almost uniformly refractory to IMV also. It is of interest to note that both patients with DHL who experienced a prolonged CR with IMV had also achieved a CR while receiving front-line therapy. Their disease-free intervals while receiving first-line therapy were 24 and 29 months, respectively.

The toxic effects of this combination are shown in tables 4 and 5. The most frequent side effect was hematuria which occurred in 33% of the patients. However, this was classified as gross hematuria in only 20% of the patients. Changes in liver enzymes and/or hyperbilirubinemia occurred in two patients who had biopsy-proven changes suggestive of drug-induced liver damage. Significant neurotoxicity in the form of moderate to severe peripheral neuropathy was also observed in 7% of patients.

The myelosuppression induced by this regimen is shown in table 5. Most of the courses were associated with acceptable myelosuppression (grades 0–2) as shown in table 5. Twenty-two percent of evaluable courses of treatment were associated with moderate to severe leukopenia (grade 3) and 18% were associated with severe thrombocytopenia. Myelosuppression occurred approximately on Days 10–14 of every course; most of the time it was of brief duration.

DISCUSSION

Most of the patients with malignant lymphoma of aggressive histologic types can achieve CR with modern combination-chemotherapy regimens (1, 11,12). However, a significant number of these patients eventually relapse from CR. The group of patients who fail to achieve CR have a grim prognosis since they uniformly develop progressive disease. The effectiveness of salvage (second-line) regimens for these histologic types has uniformly been poor. In view of such results with chemotherapy for patients with recurrent lymphoma, many physicians view secondary therapy as exclusively palliative in nature. The results of this study indicate that the IMV combination can result in prolongation of survival in relapsed patients with aggressive histologies if they achieve a CR or PR. Forty-six percent of patients with these histologic types achieved either a CR or PR. However, the most encouraging aspect of this study is the fact that two of the three patients with DHL who achieved a CR are still alive and disease-free 28+ and 33+ months after begin-

TABLE 3.—Response to IMV as related to prior response to front-line chemotherapy

Type of response to prior therapy	No. of patients	Response to IMV		
		CR	PR	Failed
CR	9	2	3	4
PR	13	3	5	5
Failed	8	0	1	7

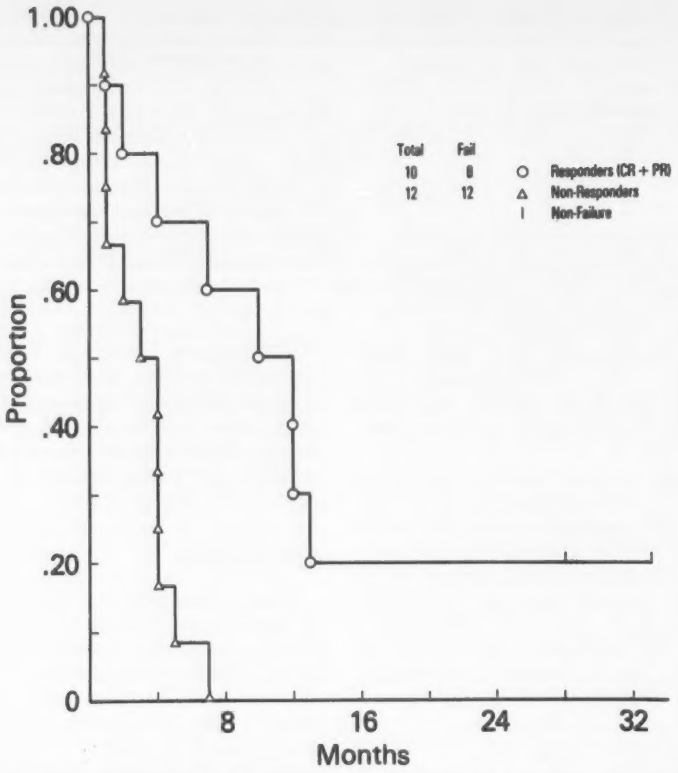

FIGURE 1.—Survival of patients with aggressive histologic subtypes according to response to therapy.

ning therapy with IMV. In the aggressive histologic types, disease-free survival of this nature is usually associated with cure in the majority of patients (1–3).

Very little can be concluded about the patients with indolent histologic types in this study due to the small numbers. However, it is also encouraging that all of the four patients who responded to the treatment with either a CR or a PR are still alive.

TABLE 4.—Malignant lymphomas: nonmyelosuppressive toxicity of IMV (total, 30 patients)

Type	No. of patients with toxicity (%)
Microscopic hematuria	4 (13)
Gross hematuria	6 (20)
Hepatotoxicity	2 (7)
Moderate to severe neurotoxicity	2 (7)

The regimen was usually well-tolerated. The most disturbing side effect was the development of gross hematuria which usually signifies series hemorrhagic cystitis. However, this has to be interpreted in view of the fact that all patients registered in this study had received cyclophosphamide, many of them for several months prior to starting ifosfamide. This compares favorably with the 40%–50% incidence of hemorrhagic cystitis reported for the single-dose schedule, mostly in patients without significant prior exposure to cyclophosphamide (13,14). The regimen might be better tolerated by patients without prior exposure to cyclophosphamide. Myelosuppression was acceptable in most of the courses administered and was always reversible, permitting subsequent courses to be modified according to toxicity.

The results of this study in patients refractory to our most active primary chemotherapy regimens, which include cyclophosphamide and doxorubicin, open the door to future use of ifosfamide

TABLE 5.—Malignant lymphomas: myelosuppression of IMV

Degree* (grade)	No. of courses with leukopenia/total No. of courses (%)	No. of courses with thrombocytopenia/total No. of courses (%)
0	6/50 (12)	25/50 (50)
1+	9/50 (18)	10/50 (20)
2+	24/50 (48)	6/50 (12)
3+	11/50 (22)	9/50 (18)

*Leukopenia (cells × 10^3/mm^3): 0 = none; 1+ = wbc count > 3.0, < 4.0; 2+ = wbc count > 1.5, < 3.0; 3+ = wbc count < 1.5. Thrombocytopenia (cells/mm^3): 0 = none; 1+ = 100,000–150,000; 2+ = 50,000–100,000; 3+ = < 50,000.

combinations in the management of lymphoma. A trial is underway at the present time in our department investigating the combination of ifosfamide, methotrexate, and VP-16-213 instead of vincristine. Combination chemotherapy with ifosfamide might also have a role in the initial management of patients with malignant lymphoma.

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Sequential Combination of Methotrexate and L-Asparaginase in the Treatment of Refractory Acute Leukemia[1]

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SUMMARY

Seventy-six patients with advanced acute leukemia refractory to conventional chemotherapy were treated with a sequential combination of methotrexate (MTX) and L-asparaginase (L-ASP), based on the reported schedule-dependent synergism between the two drugs in human leukemic cells in vitro. On Day 1, patients received 60 mg/m² of MTX iv, followed 24 hours later by L-ASP at a dose of 10,000 IU/m² iv. This sequence was repeated weekly with 50% escalations in the dose of MTX with each course. Overall, 31 of 76 patients (40.7%) achieved complete remission after a median of three courses; the response rate was 35.5% in patients with acute nonlymphocytic leukemia (21 of 59 patients) and 58.8% in patients with acute lymphocytic leukemia (ten of 17). Increasing the starting dose of MTX to 200 mg/m² did not improve the response rate. Maintenance therapy with the same combination given every 2 weeks produced a median complete remission of 10 weeks. Toxicity was manifested by: acute hypersensitivity reactions to L-ASP (five patients), stomatitis (36 patients), and mild liver abnormalities (five patients). MTX in doses up to 200 mg/m² caused minimal myelosuppression. We conclude that the MTX-L-ASP combination is a well-tolerated, highly effective induction regimen for refractory acute leukemia.

[Cancer Treat Rep 64:939–942, 1980]

The use of multiple-drug combination chemotherapy has contributed significantly to the improved prognosis of human acute leukemia (1). Recently, several papers appeared in the literature demonstrating a schedule-dependent cytotoxic synergism between methotrexate (MTX) and L-asparaginase (L-ASP) in murine leukemia cells both in vitro and in vivo (2-6), and in human leukemia cells in vitro (4,7). By giving MTX 7–14 days after a single dose of L-ASP, Capizzi (4) showed that the therapeutic effects of MTX may be enhanced with minimal toxicity. Biochemical as well as kinetic factors have been advocated to explain the improvement in the therapeutic index of MTX by L-ASP (4,6); however, the basic reasons are presently unknown. Preliminary data on the clinical effectiveness of this combination (7–10) prompted us to investigate the MTX-L-ASP regimen in the treatment of a large series of patients with advanced acute leukemia refractory to conventional chemotherapy.

MATERIALS AND METHODS

Since June 1976, 76 patients with refractory acute leukemia have been included in the study. Diagnosis of leukemia was established by bone marrow examination using accepted morphologic criteria including special cytochemical stains. Fifty-nine patients had acute nonlymphocytic leukemia (ANLL) (38 males, 21 females; median age, 22 years [range, 4–62]). Fifteen patients had never achieved an initial remission and 44 had had one or more hema-

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tologic relapses; the median duration of previous treatment was 7 months (range, 1.5–35). Previous therapy varied, but none of the 59 patients had been previously exposed either to MTX or L-ASP.

Seventeen patients had acute lymphocytic leukemia (ALL) (11 males, six females; median age, 11 years [range, 4–31]). They all had had multiple recurrences and had previously received L-ASP; 16 of the 17 patients who had previously received MTX relapsed while receiving systemic MTX as maintenance treatment in weekly doses up to 40 mg/m². The median duration of previous treatment was 29 months (range, 4–62).

The MTX-L-ASP treatment sequence was given weekly. On Day 1, patients received 60 mg/m² of MTX iv; 24 hours later, a 10,000-IU/m² dose of *Escherichia coli* L-ASP was given iv. The MTX dose was increased to tolerance by increments of 50% of the previous dose with each course, while the L-ASP dose remained constant; in 15 patients the starting dose of MTX was increased to 200 mg/m².

Cbc, hepatic, renal, pancreatic, and coagulation studies were monitored prior to each cycle; bone marrow specimens were obtained before each course of treatment. Supportive therapy with packed rbcs, platelets or granulocytes, and antibiotics were given as indicated. Therapy was administered almost exclusively on an outpatient basis; each patient was observed for at least 30 minutes for anaphylaxis after each L-ASP dose. Complete remission (CR) was defined according to the criteria of the Cancer and Leukemia Group B (11). The patients who achieved CR were asymptomatic, had no physical findings suggestive of leukemia, and had nondiagnostic bone marrow examinations and normal peripheral blood cell counts. Patients who achieved CR received the same MTX-L-ASP combination every 2 weeks as maintenance therapy, the MTX being given at the maximum-tolerated dose.

Remission and survival curves were constructed according to the method of Kaplan and Meier (12), and a generalized Wilcoxon test (13) was used to test differences between remission and survival curves. The data presented are as of September 1, 1979.

RESULTS

Overall, 31 of the 76 patients (40.7%) achieved a CR. The CR rate was 35.5% in patients with ANLL and 58.8% in those with ALL (table 1). The median time to CR in the 31 responders was 25 days, with no difference between the ANLL and ALL patients (25 versus 26 days); this represents a median of three courses of MTX-L-ASP. Seven of the 45 patients who failed to achieve a CR died during the induction phase. Six deaths were attributed to infection and one was attributed to intracranial hemorrhage.

Past responsiveness to conventional chemotherapy did not influence the subsequent response to MTX-L-ASP in patients with ANLL. The response rate in patients who had never achieved an initial remission was 33.3% (five of 15 patients), compared with 36.3% (16 of 44) in those who had had one or more hematologic relapses. In the 15 patients with ANLL who received a higher starting dose of MTX (200 mg/m²), the response rate was 26.6% (four of 15) compared with 38.6% (17 of 44) in those who had received an initial dose of 60 mg/m².

The median duration of previous treatment was 15 months (range, 1–60) for the 31 responders and 9 months (range, 1–32) for the 45 nonresponders; considering only patients with ANLL, no difference was observed in the median duration of previous treatment between responders and nonresponders (7 versus 8 months).

Twenty-eight of the 31 patients who achieved CR subsequently developed hematologic relapse. Three patients with ANLL are continuing in CR after 10–41+ weeks. The median duration of CR was 10 weeks. Neither the morphologic diagnosis nor the length of previous treatment significantly influenced the duration of response. The median survival from the start of treatment for all patients was 19 weeks, with patients who achieved CR having a median survival of 32 weeks and those who did not achieve CR having a median survival of 14 weeks.

Toxicity was manifested in the patients by: acute hypersensitivity reactions to L-ASP (five patients), mouth ulcers (36 patients), and mild liver abnor-

TABLE 1.—Results of MTX-L-ASP combination chemotherapy

Type of leukemia	No. of patients	No. of CRs (%)	No. of nonresponders (No. dying during induction)	Median duration of CR in wks (range)
ALL	17	10 (58.8)	7 (2)	9 (4–26)
ANLL	59	21 (35.5)	38 (5)	10 (4–41+)
Total	76	31 (40.7)	45 (7)	10 (4–41+)

malities (five patients). Acute hypersensitivity to L-ASP ranged from mild hives to anaphylaxis characterized by hypotension and dyspnea; there were no deaths related to acute hypersensitivity to L-ASP. Marrow hypoplasia during the induction phase was observed in 19 patients only, and was associated with infectious complications in 16 (six deaths) and bleeding episodes in three (one death). Hematologic tolerance was excellent during maintenance therapy with a dose of MTX of up to 200 mg/m². Central nervous system toxicity, pancreatitis, and hemorrhage from hypofibrinogenemia were not observed.

DISCUSSION

The results presented here are consistent with published reports suggesting an enhancement of the therapeutic index of MTX by L-ASP (table 2).

Overall, CR was induced in 40.7% of a large series of patients with advanced acute leukemia refractory to conventional chemotherapy. In particular, the CR rate for patients with ANLL was better than that achieved with other, more aggressive drug combinations used as secondary treatment (16–18). In agreement with the observations of Harris et al (10) and Yap et al (14), previous exposure to L-ASP and low-dose MTX did not preclude response to the MTX-L-ASP regimen as indicated by the 58.8% CR rate observed in patients with refractory ALL who had received both drugs during previous treatment. CR was achieved in a median of 25 days from the start of therapy after a cumulative median MTX dose of 285 mg/m².

Increasing the starting dose of MTX to 200 mg/m² did not improve the frequency of response in patients with ANLL, suggesting that the achievement of CR with MTX-L-ASP was the result of both drugs acting in concert rather than individually. The median duration of CR in the 31 responders maintained with the same combination given every 2 weeks was disappointingly short. This seems to indicate that resistance to MTX-L-ASP tends to develop quickly and that possibly better results might be obtained by combining this regimen with other drugs. In general, tolerance to the MTX-L-ASP regimen was acceptable, considering the advanced stage of the disease.

Stomatitis was the most common toxic side effect; however it was rarely a dose-limiting factor. Severe anaphylaxis occurred in only one patient and minor liver abnormalities were noted in five patients.

Bone marrow suppression was not a major problem during the induction phase, suggesting that marrow aplasia is not an absolute prerequisite for achieving CR in acute leukemia. We conclude from these results that sequential combination of MTX and L-ASP is an effective regimen for the induction of CR in patients with advanced acute leukemia; the regimen is well-tolerated, relatively nontoxic, and can be administered safely to outpatients. Enhancement of the therapeutic index of relatively high MTX doses indicates a possible rescue effect from MTX damage to normal target tissues by L-ASP.

TABLE 2.—Published reports on MTX-L-ASP induction therapy in refractory acute leukemia

No. of patients	Type of leukemia	% CR	Ref No.
13	ALL	76.9	7
3	ANLL	66.6	
23	ANLL	52.1	8
25	ANLL	20.0	9
8	ALL	75.0	10
2	ANLL	0	
21	ALL	62.0	14
7	Acute undifferentiated leukemia	43.0	
11	ANLL	18.0	
12	ALL	33.3	15
17	ALL	58.8	Present study
59	ANLL	35.5	

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Bleomycin, Vincristine, and Mitomycin C With or Without Methotrexate in the Treatment of Squamous Cell Carcinoma[1]

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SUMMARY

Forty-two patients with metastatic squamous cell carcinoma were treated with bleomycin, vincristine, and mitomycin C with or without methotrexate (BOM ± M). The overall response rate of 64% (complete response [CR] rate, 19%) included 19 responses among 26 patients (seven CRs) with head and neck cancer, three responses among eight patients with cervical cancer, and three responses among five patients (one CR) with lung cancer. Six of 12 patients (two CRs) responded to BOM and 21 of 30 patients (six CRs) responded to BOMM. The median duration of response was 16 weeks. Toxic effects included nausea or vomiting in 33% of the patients, fever of > 101°C in 26%, stomatitis in 29% and pulmonary toxicity in 19%. Four of eight cases of pulmonary toxicity were fatal and the incidence was related to the amount of both bleomycin and mitomycin C administered. The occurrence of pulmonary toxicity could not be predicted by serial determination of pulmonary function or blood gases. A wbc count nadir of < 2500/mm³ occurred in 15 of 42 patients. There were two episodes of sepsis with one death. A platelet count nadir of > 75,000/mm³ occurred in eight of 42 patients with no episodes of hemorrhage. BOMM produces a high objective response rate in patients with squamous cell cancer. However, the duration of remission is brief, and use of the regimen carries an increased risk of fatal pulmonary toxicity.

[Cancer Treat Rep 64:943–949, 1980]

Methotrexate is an established agent for the treatment of squamous cell carcinoma. It is the most effective single agent in tumors of head and neck origin (1). Although high response rates with combination-drug regimens for head and neck cancer have been reported (2–6), increased efficacy of any combination when compared to methotrexate alone has not been established in a randomized study (7–9). Cisplatin is a newer agent with activity in head and neck and cervical cancers (10–12). However, a combination regimen utilizing these two agents has a high toxicity potential since the renal toxicity of cisplatin would prevent the excretion of methotrexate and markedly increase its toxicity. Weekly or biweekly therapy with methotrexate appears most effective for producing tumor regressions (1,13). Protocols using both agents together have been forced to utilize methotrexate in a less aggressive schedule (6,14). This may decrease the therapeutic potential of the methotrexate (15). This study was done in an attempt to design a combination regimen, containing methotrexate, that did not have this potential for increased toxicity.

Bleomycin and mitomycin C are both active agents in the treatment of squamous cell carcinoma (1,16). The combination of these two agents has

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been effective in the treatment of cancer of the cervix (17,18). In addition to their therapeutic activity, the lack of bone marrow toxicity with bleomycin and the delayed marrow suppression with mitomycin C make them attractive agents to combine with methotrexate. The use of vincristine (Eli Lilly and Co, Indianapolis, IN) followed in 6–8 hours by bleomycin may increase the effectiveness of the latter agent (19).

Evidence from animal experiments and clinical trials suggests that bleomycin may be more effective when administered by continuous infusion (20–23). In addition, a low-dose bleomycin infusion may cause accumulation of cells in the G_2 phase of the cell cycle with a later synchronous wave of S phase cells when the block is released (24). Preliminary clinical trials have suggested increased antitumor effect when bleomycin and methotrexate are combined in proper sequence (25). Since the cell cycle time in head and neck cancer may extend up to 88 hours (26), we have used a 4-day infusion of bleomycin in this study. The first dose of methotrexate was delayed 40–44 hours to provide the proliferating bone marrow, which has a cell cycle time of 20–30 hours (27), time to proceed through the DNA synthetic phase while maintaining the possibility of catching the tumor population in S-phase wave. Forty-two patients with squamous cell carcinoma treated with bleomycin, vincristine, mitomycin C with or without methotrexate (BOM ± M) will be the subject of this report.

PATIENTS AND METHODS

Forty-two patients with histologically documented measurable metastatic squamous cell carcinoma, referred to the Division of Hematology/Oncology at the University of Michigan Medical Center between December 1977 and May 1979, were treated with BOM ± M. Eligibility criteria for entry in this study included measurable disease, arterial PO_2 of > 70 mm Hg on room air without CO_2 retention, a pretherapy wbc count of at least 4000/mm³, a pretherapy platelet count of at least 150,000/mm³, and a Karnofsky performance status of at least 40. Patients who had received prior therapy with bleomycin or mitomycin C were excluded. The patient characteristics including primary tumor site and prior therapy are shown in table 1. The performance status of these patients ranged from 40 to 90 with 57% having a score of ≥ 70.

The treatment regimen was as follows: vincristine (2.0 mg iv) was given on Day 1, followed in 6–8 hours by mitomycin C (15 or 20 mg/m² iv bolus), and a 4-day continuous infusion of bleomycin at a dose rate of 20 units/24 hours. Forty to 44 hours after the end of the bleomycin infusion, methotrexate (20–30 mg/m²) was given by iv push and continued weekly thereafter. The vincristine and the bleomycin infusions were repeated every 4 weeks and the mitomycin C was repeated every 8 weeks. Patients who had a creatinine clearance of < 50 ml/minute, or who had been previously treated with methotrexate, received only BOM.

The methotrexate dose was altered according to the wbc count at the time of therapy. Full dose was given if the wbc count was ≥ 4000/mm³, one-half dose was given if the wbc count was 3000–4000/mm³, and treatment was withheld if the wbc count was < 3000/mm³. A wbc count of at least 2500/mm³ was required before each bleomycin infusion, and a wbc count of 4000/mm³ and a platelet count of 150,000/mm³ were required before further mitomycin C was given. Arterial blood gases and pul-

TABLE 1.—Patient characteristics by primary site

Primary site	No. of patients	Mean age (range)	Male/female	No. of patients receiving prior therapy with— Surgery	Radiation therapy	Chemotherapy
Head and neck	26	59(47–68)	21/5	17	16	4
Tongue	8					
Pharynx	4					
Larynx	4					
Fl. mouth	2					
Tonsil	2					
Miscellaneous	6					
Lung	5	56(48–61)	3/2	2	2	2
Cervix	8	55(38–77)	0/8	3	8	6
Esophagus	2	64	0/2	1	2	—
Rectum	1	68	0/1	1	1	—
Totals	42	60(38–78)	24/18	24	29	12

monary function studies including vital capacity and a single breath carbon monoxide diffusion capacity were determined prior to each bleomycin infusion, and bleomycin was discontinued for a 10 mm Hg drop in PO_2, for CO_2 retention, or for a significant change in pulmonary function.

A complete remission (CR) was defined as the clinical disappearance of all detectable disease. A partial remission (PR) was defined as a 50% decrease in the product of perpendicular diameters of all measurable disease, and no evidence of new lesions. The duration of remission was calculated from the beginning of therapy. All patients are considered eligible for response determination.

RESULTS

The objective responses to BOM ± M are shown in table 2. The responses shown are to the drug therapy only and do not include the effect of any subsequent radiation or surgical therapies. Overall, 50% of the patients treated with BOM responded, and 17% achieved a CR. Thirty patients were treated with BOMM with an objective response rate of 70%, which includes a CR rate of 20%. Twenty-six patients in this study had primary tumors of the head and neck. Fifty-eight percent of these patients responded to BOM and 70% responded to BOMM. Six patients had far advanced, previously untreated head and neck lesions. Five of these patients achieved a PR during the chemotherapy. The three patients who had subsequent radiation therapy achieved a CR. One patient developed rapidly progressive disease during the interval between a planned two-stage surgical procedure that followed the chemotherapy and was unresponsive to further treatment. The nonresponding patient had marked regression of the primary tongue lesion and nodal metastasis, however, < 50% regression was achieved in the pulmonary metastasis. The two remaining responding patients continued to receive chemotherapy alone.

Objective regressions occurred in patients with all primary sites treated including lung, cervix, and esophagus. One patient with lung cancer refractory to CCNU and cyclophosphamide achieved a CR lasting 6 months. Overall, three of five patients with epidermoid lung cancer responded and further trials of this combination in patients with this tumor are warranted. One patient with pulmonary metastasis from carcinoma of the cervix achieved an excellent PR but died of pulmonary toxicity. At autopsy no viable tumor cells could be found and the residual lesions seen on chest roentgenograms were entirely necrotic.

Two patients achieved CR with the first bleomycin infusion cycle. Further response following the second bleomycin infusion could be determined in 17 patients and 13 showed further tumor regression with seven achieving CR in measurable disease. Two of these patients had residual unevaluable disease and have been classified as partial responders. Only one patient responded after the second bleomycin infusion after failing to show tumor regression with the first cycle, and this is the only patient who did not receive the planned methotrexate following the first infusion.

The durations of remission are shown in table 2. The four patients who had irradiation or surgery, the three patients who achieved PR but refused

TABLE 2.—Objective responses to BOM ± M

Primary site	No. of patients	No. of objective responses (%)		Duration of remission in wks (response type)
		CR	PR	
BOM				
Head and neck	7	2(29)	2(29)	18,27 (CR)
Cervix	3		1	10 (PR)
Lung	1		1	10 (PR)
Esophagus	1			
BOMM				
Head and neck	19	5(26)	10(53)	10*,10*,13,16,30+ (CR) 15,16*,17,18 (PR)
Cervix	5		2	11*,16 (PR)
Lung	4	1	1	24 (CR) 11 (PR)
Esophagus	1		1	9* (PR)
Rectum	1		1	15 (PR)
Totals	42	8(19)	19(45)	

*Patient died in remission.

further therapy, and the one patient who was found to have a cholangiocarcinoma of the liver are not included. Five patients died while in remission, four of pulmonary toxicity and one of sepsis during drug-induced bone marrow suppression. In general the PRs were of short duration with a median of 16 weeks. The median duration of remission in complete responders who did not die of drug-induced toxicity is 22 weeks, with one patient still in remission. Most patients recurred in areas of known disease at the time treatment was initiated. The duration of CRs and PRs are similar and probably reflect the difficulty in assessing tumor status in patients with head and neck cancer who have had surgical and irradiation therapy. CR was documented by biopsy in two patients. However, both patients relapsed and died of recurrent disease shortly thereafter. Although we plan to histologically document CRs in future studies, biopsies in areas of previous irradiation will probably have considerable sampling error. The poor correlation between clinical CR, biopsy confirmation, and subsequent surgical findings has been recently reported by Hong et al (3).

The toxicity experienced with BOM ± M is shown in table 3. The first nine patients treated received 20 mg/m² of mitomycin C. Two patients received 20 mg/m² of methotrexate and seven received 30 mg/m². Analysis of these patients showed that only about one half of the planned methotrexate was being delivered. The mitomycin C dose was then decreased to 15 mg/m² for subsequent patients with an increase in the total amount of methotrexate administered. The overall hematologic toxicity was quite tolerable. Two patients mistakenly received the first methotrexate injection at the end of the bleomycin infusion despite a low wbc count. Both patients developed marked leukothrombocytopenia and sepsis which was fatal in one patient. No other episodes of drug-associated infections occurred. There were no episodes of life-threatening hematologic toxic effects in the patients treated with BOM.

Gastrointestinal toxicity was mild. The nausea and occasional vomiting noted was controlled by antiemetics. Fever > 101°C occurred in 11 patients, usually within the first 24 hours of the bleomycin infusion. It was rarely sustained or symptomatic. Three patients developed a diffuse, erythematous rash following the bleomycin infusion, and corticosteroids were administered in two instances.

We had initially planned to give a total of four bleomycin infusions 4 weeks apart, and to continue the mitomycin C every 8 weeks. However, as shown in table 4, of the first seven patients to receive the second dose of mitomycin C and a third bleomycin infusion, three developed rapidly progressive, fatal pulmonary toxic effects. Thereafter, only two bleomycin infusions were administered. The next six patients received only two bleomycin infusions followed by a second dose of mitomycin C. Three of these six patients developed pulmonary infiltrates, one asymptomatic and two symptomatic, but all gradually resolving. We have therefore reduced the treatment program to one mitomycin C dose and two bleomycin infusions, and in the next 29 patients only two episodes of pulmonary toxicity have occurred. One patient with head and neck cancer de-

TABLE 3.—Toxicity of BOM ± M

		BOMM (total: 30 patients)	
	BOM (total: 12 patients)	Mitomycin C (15 mg/m²) (21)	Mitomycin C (20 mg/m²) (9)
Hematologic nadir			
Median wbc count (cells × 10^3/mm³)	3.7 (1.5–8.1)*	3.0 (0.1–5.7)	2.7 (0.1–4.0)
Median platelet count (cells × 10^3/mm³)	205 (27–348)	130 (9–287)	86 (30–230)
No. of patients with—			
Gastrointestinal			
Nausea and vomiting	4	10	
Stomatitis	2	10	
Fever	4	7	
Skin	1	2	
Pulmonary	1	7	

*Nos. in parentheses = range.

TABLE 4.—Pulmonary toxicity of BOM ± M

No. of bleomycin infusions (80 units/infusion)	No. of mitomycin C doses (15–20 mg/m²)	No. of patients with toxic effects/No. treated			
		Pulmonary toxicity			
		BOM	BOMM	Total	Fatal
1	1	0/3	0/4	0/7	—
2	1	0/8	2/12	2/20	1/2
2	2	1/1	2/5	3/6	0/3
3	2	—	3/7	3/7	3/3

veloped minimal, asymptomatic basilar infiltrates on chest roentgenogram. A second patient with esophageal carcinoma who had received radiation therapy to the primary tumor developed pulmonary toxic effects following the second bleomycin infusion that partially contributed to her death.

DISCUSSION

BOMM has activity in squamous cell carcinoma arising in a number of primary sites. The objective response rate of tumors in the head and neck is particularly encouraging in view of a 60% response rate, including a 35% CR rate that occurred in the 20 patients who had been previously treated with irradiation and/or surgery. Previously irradiated patients have been shown to have a lower response rate to both intraarterial and systemic chemotherapy (2,5,28).

To date, randomized trials have not shown any drug combination or high-dose methotrexate with citrovorum factor rescue to be superior to methotrexate alone (7–9). Weekly or biweekly methotrexate has produced tumor regression in 50% of patients treated (1). Response rates of 15%–36% have been reported for single-drug therapy with bleomycin, doxorubicin, cyclophosphamide, 5-FU, and mitomycin C (1,16). Cisplatin has induced remissions in 31% of treated patients (11,12). Response rates similar to BOMM have been shown for cisplatin plus bleomycin with or without the addition of methotrexate with citrovorum factor rescue, and high-dose methotrexate with citrovorum rescue (2,5,29). In all of these studies the higher response rate occurred in patients who have not been previously irradiated. A 61% response rate has been recently reported for the combination of bleomycin, cisplatin, and methotrexate (6). Twenty-three percent of the patients in this series achieved CRs. The duration of remission is impressive and will probably exceed 1 year. Any superiority of BOMM compared to these other programs would have to be established by a randomized trial. Although the number of patients who received BOM alone is small, the 58% response rate demonstrates that this part of the combined-treatment regimen has activity independent of the methotrexate.

BOM ± M produced tumor regression in all primary tumor sites treated. Three of five patients with lung cancer responded, one with a CR lasting 6 months. Three of eight patients with carcinoma of the cervix had PRs. One of two patients with cancer of the esophagus also responded, as did one patient with squamous cell carcinoma of the rectum.

Previous in vitro and human studies have shown that continuous exposure to low concentrations of bleomycin will produce cell accumulation in the G_2 phase of the cell cycle (29), and that a synchronous wave of DNA synthesis will follow release of the block. Preliminary studies using methotrexate following a bleomycin infusion have shown a high response rate in head and neck cancer (25). Although the BOMM program was designed with such cytokinetic data in mind, the success of the program is not necessarily a result of this attempt at cell synchrony. Simultaneous cytokinetic analysis would be required to demonstrate the efficacy of this approach (27).

Gastrointestinal side effects and stomatitis were mild when they occurred. Except for two patients who mistakenly received the first dose of methotrexate despite depressed wbc counts, no life threatening hematologic toxicity occurred. Surprising, however, was the severe pulmonary toxicity encountered early in the program. Three of seven patients who received a third bleomycin infusion and a second dose of mitomycin C had fatal pulmonary fibrosis. The dose-related lung toxicity of bleomycin has been well-described (30). The cumulative bleomycin dose was 240 units in these seven patients. Although fatal pulmonary toxicity has been reported to occur with cumulative doses of < 200 units (31), only a 10% incidence of pulmonary toxicity would be expected at this dose (29). Clinical trials and animal experiments have not demonstrated increased toxicity for infusional therapy (20,22,23,32). An age-related increase in toxicity has been noted (29). However, all four patients

who developed pulmonary toxicity were < 70 years old and two were < 60 years old.

Serial determination of pulmonary function has been suggested as a potential method of avoiding bleomycin pulmonary toxicity (30). Arterial blood gases were determined in all four patients who developed fatal lung toxic effects prior to the final bleomycin infusion, and sequential pulmonary function studies were obtained in three patients. There was no demonstrable change from pretherapy studies in arterial gas tensions, physical examination, patient symptoms, or diffusion capacity prior to the onset of overt pulmonary toxicity.

Mitomycin C pulmonary toxicity has been described (33), as has lung toxicity due to methotrexate (34,35). The toxicity seen with this program is probably the result of the addition of either or both of these agents to the bleomycin infusion. Methotrexate lung toxicity has usually been accompanied by high fever and has, in general, resolved with or without corticosteroid administration (33,34). Fever was not seen in these patients, and none of the patients who died responded to corticosteroids. The contribution of mitomycin C is suggested by the three patients who developed nonfatal pulmonary infiltrates following a second dose of mitomycin C without further bleomycin. Only two episodes of pulmonary toxicity were seen after the bleomycin and mitomycin C was limited to two infusions and one dose, respectively. Minimal asymptomatic basilar infiltrates were noted in the chest roentgenogram of one patient who also had disease progression. Fatal pulmonary toxic effects occurred in a 66-year-old patient with esophageal cancer who had received irradiation to the primary tumor.

BOMM has a high response rate in patients with squamous cell cancer, particularly of head and neck origin. With appropriate dose limitation it is well tolerated in the majority of patients. There is, however, a considerable risk of fatal pulmonary toxicity when more than two bleomycin infusions are administered. The duration of remission and CR rate are disappointingly low. The high initial response rate suggests that it might best be used as an induction regimen prior to planned surgery, irradiation, or a cisplatin-containing consolidation program. These uses are currently under study.

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Phase I Trial of Metoprine in Patients With Advanced Cancer[1,2]

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SUMMARY

The effects of metoprine administered orally every 2 weeks were studied in 71 evaluable adult patients with advanced malignant tumors. Two escalating dose schedules were explored in this phase I evaluation: (*a*) doses ranging from 20 to 65 mg/m² without scheduled leucovorin, and (*b*) doses ranging from 100 to 300 mg/m² with scheduled iv leucovorin. Thrombocytopenia was dose-limiting at 65 mg/m² in the low-dose schedule; CNS toxicity was dose-limiting at 300 mg/m² in the high-dose schedule. Occasionally leukopenia and mild nausea or vomiting were noted. Therapeutic responses were observed in patients with mycosis fungoides, non-Hodgkin's lymphoma, and adenocarcinoma of unknown origin. Phase II (disease-oriented) studies can appropriately be initiated with fortnightly metoprine at 50 mg/m² without leucovorin. In the high-dose schedule, 225 mg/m² of metoprine with 75 mg/m² of iv leucovorin at 24 hours appears appropriate for good-risk patients; in marginal-risk patients, two doses of leucovorin should be given: 75 and 37.5 mg/m² at 24 and 96 or 168 hours, respectively.

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Metoprine (fig 1) is a potent lipid-soluble inhibitor of dihydrofolate reductase (1). The drug entered clinical trial in 1953 after antitumor activity was demonstrated against Sarcoma 180 (2) in both methotrexate-sensitive and -resistant strains of murine AK4 and AK4R leukemias (3). In these early clinical trials metoprine had antitumor activity in patients with acute leukemia (4), but the study, which was based on administration of the drug daily until toxicity developed, was abandoned because the toxicity induced was severe and prolonged. More recent studies done by Cavallito et al (5) demonstrated that metoprine had a prolonged plasma half-life in man, with a mean of 216 hours and a range of 165–268 hours. Retrospectively, the prolonged drug toxicity observed in the initial clinical studies is now understandable in terms of the prolonged plasma and tissue retention of metoprine. The drug is presently being re-evaluated clinically in light of this new understanding of its pharmacokinetic characteristics.

In recently initiated studies in Europe, metoprine has been given in large intermittent doses which require scheduled administration of leucovorin to prevent serious toxicity. Price et al (6) have reported that metoprine produced useful antitumor effects in patients with lung cancer, melanoma, and hypernephroma. Alberto et al (7) have reported effects in patients with cancer of the lung, head and neck, or cervix.

Although Sirotnak et al (8), in further studies of the Sarcoma 180 model, observed that the optimal therapeutic index of metoprine was achieved by intermittent dose schedule followed by leucovorin at 24 hours, an optimal clinical dose schedule for this drug has not been established. The purpose of the present study was to develop two schedules that

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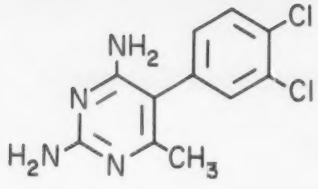

2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine

FIGURE 1.—Structure of metoprine.

would be suitable for phase II use and would permit comparison of the therapeutic utility of (*a*) a low-dose schedule without leucovorin, and (*b*) a high-dose schedule with leucovorin given in a manner planned to prevent excessive toxicity. The results of this phase I study are reported here.

PATIENTS AND METHODS

Patients who had histologic evidence of advanced malignancy and for whom standard therapy was not indicated were included in this study. Each patient had a performance status of ≥ 50 (Karnofsky scale) and an estimated survival of ≥ 8 weeks. No patient had received other therapy for ≥ 4 weeks prior to receiving metoprine. All patients had acceptable hematologic status (wbc count of ≥ 4000/mm³ and platelet count of ≥ 120,000/mm³ as well as a bilirubin level of ≤ 1.5 mg/dl and a serum creatinine level of ≤ 2.0 mg/100 ml). Informed consent for the drug trial was obtained for all participants.

Metoprine was supplied as 2.5-, 10-, and 50-mg tablets by Wellcome Research Laboratories, Burroughs Wellcome Co (Research Triangle Park, NC). Leucovorin was supplied as the powder by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD; it was reconstituted with sterile water to a concentration of 10 mg/ml and given by rapid iv injection. In a course of therapy at a given dose level, that particular dose was given every other week for three doses. The *low-dose schedule* of metoprine was initiated at 20 mg/m². In the absence of dose-limiting toxicity in the group of patients treated at a given dose level, the higher level was implemented at increments of 10–15 mg/m² until the maximally tolerated level was reached (ie, 65 mg/m²). In the *high-dose schedule*, the initial metoprine dose was 100 mg/m²; the dose was escalated until the toxic dose high was reached at 300 mg/m². Leucovorin was given by slow iv push at 24 and 96 hours; on a milligram-per-square-meter basis, each leucovorin dose was one third the metoprine dose. Another high-dose regimen was explored, keeping the dose of metoprine constant at 225 mg/m² and de-escalating the dose of leucovorin.

Prior to the start of chemotherapy, all patients had a complete history and physical examination, laboratory evaluation (including cbc with differential and platelet counts, 12-channel screening profile, 5'-nucleotidase, serum electrolytes and glucose, prothrombin time, serum creatinine, and urinalysis), electrocardiogram, and chest roentgenogram. All patients were monitored for evidence of hematopoietic, gastrointestinal, hepatic, renal, and CNS toxicity. Weekly followup studies included cbc and platelet count, 12-channel screening profile, 5'-nucleotidase, and serum creatinine. A chest roentgenogram was done monthly.

Response criteria were defined as: *complete response (CR)*—the disappearance of all clinical evidence of active tumor for ≥ 4 weeks, and patients free of all symptoms; *partial response (PR)*—a 50% decrease in the sum of the products of the greatest perpendicular diameters of all measurable lesions without evidence of increase in the size of any lesion or the appearance of new lesions; *minor response*—an objective (≥ 25%) decrease in tumor size, but less than that required for PR; *no change*—a < 25% decrease in tumor size; and *disease progression*—an objective increase (> 25%) in measurable disease or the appearance of new tumor lesions.

RESULTS

Myelosuppression proved to be the dose-limiting toxic effect in the low-dose regimen of this phase I trial. Thrombocytopenia was the most frequently observed hematologic effect of metoprine (tables 1–3). We cannot account for the myelosuppression seen at the 40-mg/m² dose level since it was not dose-limiting, as attested by the results of the 50-mg/m² dose (table 4). Four of ten courses (40%) at a dose of 65 mg/m² resulted in platelet count depression to ≤ 30,000/mm³; all four patients required platelet transfusions and leucovorin rescue for minor but persistent hemorrhage. Blood cell count depression was generally maximal 2–4 weeks after initiation of metoprine, with recovery of the cell counts in 7–10 days.

Leukopenia occurred less frequently than thrombocytopenia. While some patients had a wbc count nadir of < 4000/mm³, a median nadir of < 4000/mm³ was never reached (table 4). None of the instances of leukopenia resulted in septicemia. Anemia related to metoprine occurred occasionally in those patients experiencing marked leukopenia and thrombocytopenia.

TABLE 1.—Hematopoietic toxicity of metoprine therapy without leucovorin: dose-effect relationships

Metoprine dose (mg/m^2)*	No. of patients at risk	No. of evaluable patients	No. of patients with wbc count nadir (cells × $10^3/mm^3$) of—			No. of patients with platelet count nadir (cells × $10^3/mm^3$) of—		
			⩾ 4.0	2.0–3.9	< 2.0	> 150	80–150	< 80
20	5	5	4	1	0	3	1	1
30	17	15	9	6	0	8	2	5
40	12	10	5	2	3	3	1	6
50	10	8	6	0	2	6	1	1
65	10	10	5	2	3	2	2	6

*Dose given q2wks × 3 (1 course) unless toxicity occurred earlier.

TABLE 2.—Hematopoietic toxicity of metoprine with leucovorin*: dose-effect relationships

Metoprine dose (mg/m^2)	No. of patients at risk	No. of evaluable patients	No. of patients with wbc count nadir (cells × $10^3/mm^3$) of—			No. of patients with platelet count nadir (cells × $10^3/mm^3$) of—		
			⩾ 4.0	2.0–3.9	< 2.0	> 150	80–150	< 80
100	3	3	3	0	0	3	0	0
125	4	4	4	0	0	4	0	0
175	7	7	6	1	0	4	0	3
225	6	5	5	0	0	3	2	0
300	8	7	5	0	2	3	2	2

*Leucovorin dose: ⅓ dose of metoprine given iv at 24 and 96 hrs.

TABLE 3.—Hematopoietic toxicity of metoprine at a dose of 225 mg/m^2 with decreasing doses of leucovorin: dose-effect relationships

Leucovorin dose (mg/m^2) at—		No. of patients at risk	No. of evaluable patients	No. of patients with wbc count nadir (cells × $10^3/mm^3$) of—			No. of patients with platelet count nadir (cells × $10^3/mm^3$) of—		
24 hrs	96 hrs			⩾ 4.0	2.0–3.9	< 2.0	> 150	80–150	< 80
75	75	6	5	5	0	0	3	2	0
75	37.5	12	10	8	1	1	5	2	3
75	0	10	6	3	3	0	1	2	3

CNS toxicity proved dose-limiting in patients receiving the high-dose regimen with two equal iv doses of leucovorin, ie, one third of the metoprine dose at 24 and 96 hours. Five of the seven patients receiving the 300-mg/m^2 dose experienced neurologic toxic effects within 3–12 hours of receiving metoprine: hallucinations (two patients), focal seizures (one), and hyperactivity and insomnia (two). Although the symptoms markedly abated within 6 hours after iv leucovorin (at 24 hours) was given, they were sufficiently severe prior to the leucovorin that no additional patients were entered into the study at the 300-mg/m^2 level.

Metoprine at a dose of 225 mg/m^2, with 75 mg/m^2 of iv leucovorin at 24 and 96 hours, was the maximal tolerated dose with regard to CNS effects; it did not produce consistent myelosuppression. The 225-mg/m^2 dose of metoprine was maintained but leucovorin was attenuated to one third at 24 hours and at 96 hours and then one third the metoprine dose at 24 hours only. With these modifications, the low-dose (50 mg/m^2) metoprine every 14 days without leucovorin and the high-dose (225 mg/m^2) metoprine with iv leucovorin at 24 and 96 hours produced comparable and acceptable thrombocytopenia.

Myelosuppression did not become cumulative in the high-dose (plus leucovorin) schedule given every 14 days. In four patients the second dose of leucovorin was given on Day 7, a more convenient outpatient schedule, without increasing toxicity. Patients receiving high-dose metoprine required further leucovorin at 14 or 15 days even if further metoprine was not given, presumably because of the prolonged retention of metoprine.

TABLE 4.—Metoprine toxicity with and without leucovorin

Metoprine dose (mg/m²)	No. of patients at risk	No. of evaluable patients	Hematopoietic toxicity: Wbc count Nadir range	Wbc count Median nadir (Day)	Platelet count Nadir range	Platelet count Median nadir (Day)
Without leucovorin						
20	5	5	3.3–32.4	4.5 (8)	27–283	156 (29)
30	17	15	2.7–19.2	5.1 (26)	14–358	184 (29)
40	12	10	0.7–12.5	3.9 (20)	18–320	48 (24)
50	10	8	1.0–13.9	6.1 (32)	10–261	164 (49)
65	10	10	0.8–6.8	3.8 (13)	6–191	44 (15)
With leucovorin CF#1*						
100	3	3	4.6–12.6	5.1 (1)	190–370	285 (28)
125	4	4	4.4–12.6	5.1 (27)	180–307	285 (27)
175	7	7	3.1–10.7	6.2 (5)	19–453	165 (24)
225	6	5	4.2–16.3	8.4 (10)	100–467	259 (10)
300	8	7	1.5–9.9	4.7 (22)	23–265	130 (26)
With variable leucovorin*						
225/CF#1	6	5	4.2–16.3	8.4 (10)	100–467	259 (10)
225/CF#2	12	10	1.4–9.4	6.2 (13)	10–340	157 (15)
225/CF#3	10	6	2.1–38.4	4.0 (20)	14–182	72 (17)

*CF#1 = ⅓ metoprine dose at 24 and 96 hrs. CF#2 = ⅓ metoprine dose at 24 and ⅙ at 96–168 hrs. CF#3 = ⅓ metoprine dose at 24 hrs only.

Acute side effects of metoprine were generally mild and infrequent. Only 15 of the 71 patients had nausea; seven had vomiting. Anorexia was common at the 225- and 300-mg/m² dose levels. No patient in this trial experienced drug-associated hepatic dysfunction or azotemia as determined by serial SGOT, bilirubin, 5′-nucleotidase, alkaline phosphatase, BUN, and serum creatinine.

Because drug-resistant leukemic patients with full marrow and peripheral relapse commonly require relatively massive doses of antimetabolites to produce tumor cell-kill, the leucovorin rescue was delayed in two such patients in order to avoid rescuing the leukemia. Both patients received 225 mg/m² of metoprine while leucovorin was withheld until Day 5 and Day 10, respectively. These patients experienced *continuous* CNS toxicity for 5 and 10 days with hallucinations and hyperactivity until iv leucovorin was administered. They also had severe cutaneous toxic effects that were reversed by leucovorin in the patient rescued at 5 days.

Seventy-one of the 83 patients entered in this trial were evaluable for toxic effects. Six patients were considered inadequately treated due to early deaths from disease; two patients refused completion of the three-dose course of drug treatment. There were four incidents of protocol violation.

Six patients were judged to have experienced clinically useful therapeutic response (CR plus PR) to metoprine (table 5). A CR lasting 11+ months was achieved by one patient with mycosis fungoides who received 175 mg/m² of metoprine with leucovorin. PRs were achieved by three other patients with the same diagnosis who were treated with 225 mg/m² of metoprine plus leucovorin; their remissions lasted 2, 2, and 5 months. A PR of 3+ months was also achieved in one patient with non-Hodgkin's lymphoma who was treated with 225 mg/m² of metoprine with leucovorin, and a PR of 8+ months was achieved in one patient with adenocarcinoma of unknown primary origin who received 65 mg/m² of metoprine without leucovorin (low-dose schedule).

DISCUSSION

This further clinical investigation of metoprine was undertaken to develop two schedules that would produce comparable toxicity: (*a*) low-dose metoprine without leucovorin and (*b*) high-dose metoprine with leucovorin. The purpose of this approach was to permit a prospective comparison in disease-oriented phase II studies of these schedules, thus rigorously examining whether the high-dose rescue technique had a better therapeutic index than the simpler low-dose regimen. The choice of drug administration at 2-week rather than 3-week intervals was arbitrary but practical on an outpatient basis.

Disease-oriented phase II studies are planned at this Center for several tumor varieties using high-dose versus low-dose metoprine with scheduled leucovorin. With the acceptability and manageability

TABLE 5.—Summary of response to metoprine

Diagnosis	No. of evaluable patients	Response category				
		CR*	PR*	Minor response	No change	Disease progression
Non-Hodgkin's lymphoma	5	0	1 (3+)	1	0	3
Hypernephroma	9	0	0	1	1	7
Esophageal carcinoma	2	0	0	0	0	2
Breast carcinoma	4	0	0	0	0	4
Cervical carcinoma	2	0	0	1	0	1
Pancreatic carcinoma	1	0	0	0	0	1
Adenocarcinoma (unknown primary)	5	0	1 (8)	0	1	3
Colon carcinoma	1	0	0	0	0	1
Head and neck carcinoma	3	0	0	0	0	3
Lung carcinoma	23	0	0	1	3	19
Mycosis fungoides	11	1 (11+)	3 (2,2,5)	6	0	1
Sarcoma	2	0	0	0	1	1
Epidermoid skin carcinoma	1	0	0	0	1	0
Acute leukemia	2	0	0	0	0	2
Total	71	1	5	10	7	48

of the observed toxicity, we suggest a phase II administration schedule in the low dose at 50 mg/m² every 2 weeks, escalating the dose to 55 mg/m² and then to 60 mg/m² if there is no hematologic toxicity in 4 weeks. In the high-dose regimen with scheduled leucovorin rescue, we recommend a dose of 225 mg/m² every 2 weeks with 75 mg/m² of leucovorin at 24 hours and 37.5 mg/m² at 96 or 168 hours; if no hematologic toxicity is observed, the 96-hour leucovorin may be discontinued. With these adjustments the two treatment groups may experience comparable myelosuppression. Only the high-dose group will experience CNS side effects, but these are acceptable to patients, provided rescue is given at 24 hours.

Although the CNS toxicity of metoprine remains unexplained, our data suggest that it can be blocked and reversed by leucovorin in man. Sirotnak et al (unpublished data) have recently re-examined the acute convulsive CNS death produced by $BD2F_1$ mice by ip metoprine. They find that the CNS death can be prevented by adequate doses of leucovorin administered prior to metoprine. Although barbiturates at anticonvulsive doses can also prevent metoprine-induced convulsions, protection by leucovorin, an agent without significant sedative actions, implies the existence of a toxic mechanism related to some physiologic function of reduced folate. Acute CNS stimulation of metoprine manifested by rapidity of thought, hyperactivity, insomnia, headache, nausea, and vomiting was repeatedly observed by Brenckman and co-workers (personal communication) who found that a single metoprine dose of 120 mg/m² gave unacceptable CNS toxicity in their patients who were receiving leucovorin rescue by an oral low dose of 15 mg at 48, 72, and 96 hours. This dose prevented intestinal and marrow toxicity.

In contrast to Brenckman's experience, Price et al (6) did not observe serious CNS effects of metoprine when simultaneously administering oral leucovorin in doses comparable to those used in our studies. Other antifolates do not produce acute neurotoxicity. Methotrexate does produce CNS injury when given by the intrathecal route or administered repeatedly iv to patients who have also received whole-brain irradiation (9–12). This injury, however, is generally a late manifestation in continuously treated patients.

The above considerations do not exclude the possibility that altered brain histamine metabolism may also play a role in the observed CNS effects of metoprine. Duch et al (13) observed that metoprine was a potent inhibitor of histamine *N*-methyl transferase, an enzyme that participates in histamine degradation. They postulated that metoprine therapy led to a buildup of brain histamine levels, thereby producing the observed toxic effect.

In view of Brenckman's experience, we were somewhat surprised to find that we could escalate the metoprine dose to 300 mg/m² before we obtained consistent dose-limiting effects on the brain. Our patients were moderately restless for 18 hours, but the symptoms promptly abated in most instances after the patient received iv leucovorin. Only when we delayed leucovorin in an effort to obtain a more adequate antileukemic effect in the two patients with acute leukemia did we observe CNS effects of

magnitude comparable to those Brenckman had reported. One of the patients with leukemia had some weeks previously received the same dose of metoprine with leucovorin rescue at 24 hours without experiencing either CNS toxic effects or antileukemic effect. The available clinical data seem reconcilable with a CNS toxicity mechanism wherein leucovorin (or 5-methyltetrahydrofolate) produces reversal or protection. The mechanism by which this protection is achieved remains obscure.

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Results of Whole-Brain Irradiation for Metastases From Small Cell Carcinoma of the Lung[1]

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Summary

Prophylactic brain irradiation significantly reduces the proportion of patients with small cell carcinoma of the lung (SCCL) who develop brain metastases. Therapeutic brain irradiation could be used instead of prophylactic irradiation if it could be delivered promptly after diagnosis of intracranial spread and if it were highly effective. Forty of 188 patients with SCCL seen in the Medical College of Wisconsin Affiliated Hospitals had brain metastases either at initial diagnosis (23 patients) or after treatment (17 patients). Irradiation was administered to the whole brain at doses ranging from 3000 rads in 2 weeks to 4000 rads in 3 weeks. Neurologic function was the same for those patients with initial and delayed metastases and did not correlate with survival. Response to irradiation affected survival. After the diagnosis of brain metastases, survival was the same whether metastases were initially present or were delayed. Eighteen (45%) of the patients died from brain metastases and two (5%) are alive following disease recurrence in the brain. Increasing survival with SCCL due to effective chemotherapy is associated with an increasing failure rate in the brain which may eventually reach the 50% rate found at autopsy. Therapeutic brain irradiation is not sufficiently effective to preclude prophylactic irradiation.

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Prophylactic irradiation of the brain for presumed micrometastases from small cell carcinoma of the lung (SCCL) has been adopted as standard practice in many institutions in the US. Although irradiation to low total doses (2000 rads/10 fractions/2 weeks) has little or no effect (1), higher doses (3000 rads/10 fractions/2 weeks), with systemic chemotherapy, have reduced the clinical occurrence of brain metastasis from 20%–25% (1,2) to 5% (3).

Considering the striking responsiveness of this tumor to irradiation, it is conceivable that treatment could be withheld until clinical evidence of intracranial metastasis becomes apparent. The treatment would have to be prompt, highly effective, and free of serious side effects in order to rival the efficacy of prophylactic irradiation. If therapeutic brain irradiation were sufficient, prophylactic irradiation would be unnecessary—not only for the high proportion of patients who might never manifest brain metastases, but also for those who would.

The present study was undertaken to evaluate therapeutic brain irradiation for patients with SCCL. Thus, the use of prophylactic irradiation could be placed in perspective in the overall management of this disease.

PATIENTS AND METHODS

The records of all patients with SCCL who were seen in the Division of Therapeutic Radiology of the Medical College of Wisconsin Affiliated Hospitals (MCWAH) were searched from 1974 through June

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1979. Those patients who were found to have intracranial metastases, either at presentation or following initial therapy, were selected for detailed review. Radionuclide scans and/or computed tomograms (CT), in addition to symptoms and signs, were used to determine brain metastases. Based upon clinical findings at the time of diagnosis of brain metastasis, the patients were retrospectively assigned to one of four categories of neurologic function (NF) as follows:

1. able to work, minor neurologic findings;
2. able to be home, nursing care may be required, moderate neurologic findings;
3. necessary hospitalization, major neurologic findings; and
4. hospitalization necessary, serious neurologic findings: coma.

The entire brain was treated with cobalt-60 gamma rays or with 4-, 6-, or 25-MV X-rays. The response to irradiation was categorized as follows: *complete response (CR)*—the disappearance of all neurologic symptoms and signs; *partial response (PR)*—improvement in NF, but definite symptoms and signs persist; and *no change or progression*—neurologic symptoms and signs stable or deteriorating. Brain and CT scans were not used to determine response since the interpretation of the changes after treatment is controversial. Survival curves were determined by the actuarial method recommended by the American Joint Committee for Cancer Staging and End-Results Reporting (4).

RESULTS

During the 66 months of study, 188 patients with SCCL were seen in the Division of Therapeutic Radiology, MCWAH. Forty patients (21%) were found to have brain metastases. They ranged in age from 44 to 85 years, with a median of 60 years. There were 31 men and nine women.

The intervals from the diagnosis of SCCL to the appearance of brain metastases are shown in table 1. Twenty-three patients were found to have brain metastasis within 10 days of the initial presentation

TABLE 1.—Interval between the initial diagnosis of SCCL and brain metastasis

Days	No. of patients
0–10	23
11–100	4
101–300	6
> 300*	7
Total	40

*Longest: 706 days.

TABLE 2.—NF category at diagnosis, as related to interval between diagnosis of primary tumor and brain metastasis

NF category	No. of patients with—	
	0–10-day interval	> 10-day interval
1	5*	2
2	8	5
3	6	6
4	1	4
Totals	23	17

*5 patients were asymptomatic; diagnosis was done by scans.

(including five asymptomatic patients in whom the metastases were found by systematic initial evaluation with brain scans), and 17 patients had delayed manifestations of intracranial disease after treatment. In this latter group, treatment had consisted of thoracic irradiation alone for six patients, chemotherapy alone for one, and local irradiation plus chemotherapy for ten. Previous therapy did not include prophylactic irradiation of the brain in any of the patients. Seven patients developed brain metastases > 1 year after diagnosis, and the longest interval was nearly 2 years (706 days).

The diagnosis of brain metastasis was made by radionuclide scan (25 patients), CT (six), or both (three). Six patients had normal radionuclide scans but had progressive neurologic signs at a time when CT was not available; metastases were confirmed by arteriography, biopsy, or autopsy.

The 35 symptomatic patients had headache (11 patients), confusion (11), paresis (nine), seizures (six), ataxia or incoordination (four), blurred vision or a visual defect (four), cranial nerve findings (four), and expressive aphasia (two). NF prior to brain irradiation is shown in table 2. Although a higher proportion of patients with initial brain involvement were in the NF 1–2 categories (16 of 23 patients) than those with delayed metastases (seven of 17), the differences between the initial and delayed group are not significant ($P > 0.05$).

Fifteen patients (37.5%) had clinical CRs to whole-brain irradiation (scans were not systematically repeated). PRs were seen in another 15 patients (37.5%). Ten patients (25%) had no change in NF or had progressive deterioration. Eight of the NC patients, four of 23 from the initial group and four of 17 from the delayed group, failed to complete the planned course of whole-brain irradiation. The doses administered to the midsagittal plane of the patients who completed cranial irradiation ranged from 3000 rads in 2 weeks to 4000 rads in 4 weeks. Survival correlated better with the response to irradiation (fig 1), than it did with NF

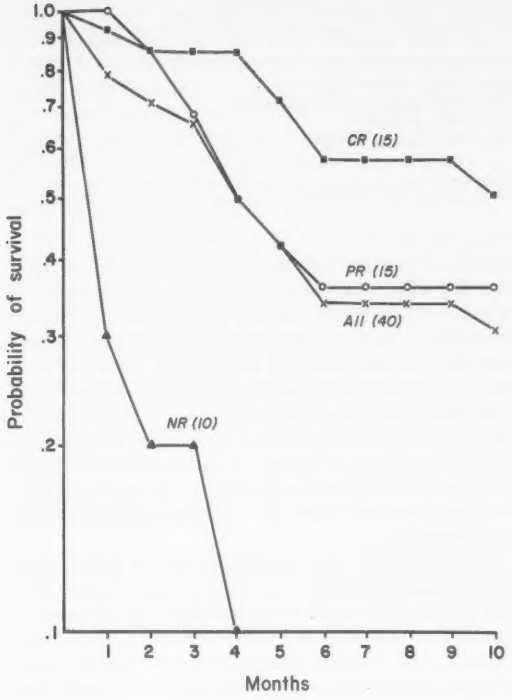

FIGURE 1.—Survival from diagnosis of brain metastases by response to irradiation.

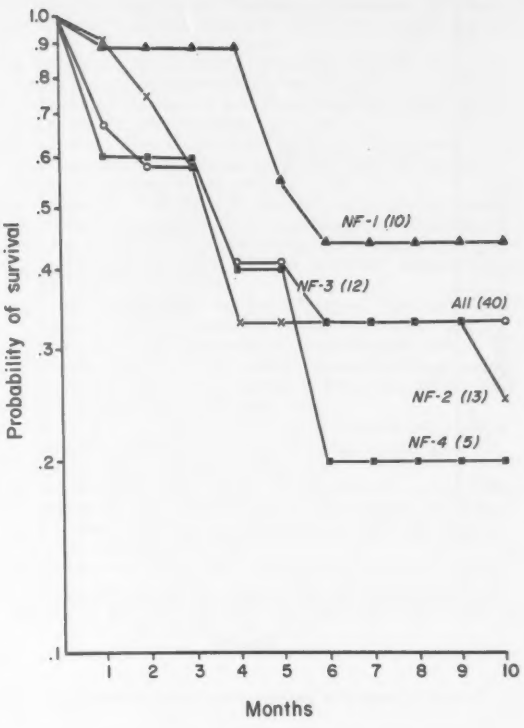

FIGURE 2.—Survival by NF category.

at the time of the diagnosis of brain involvement (fig 2).

Seven patients lived ≥ 12 months after irradiation for intracranial disease. Four of these patients had brain involvement and received thoracic irradiation and systemic combination chemotherapy in addition to the cranial irradiation. The longest survival after the diagnosis of SCCL was 27 months, but this patient died with progression of intracranial metastasis 4 months after irradiation of the brain. The longest survival after the diagnosis of brain metastasis was 21 months. Survival from the date of brain metastasis was the same for those patients who had intracranial involvement at presentation and those who had delayed brain involvement (fig 3).

The current status of the 40 patients included in this series is shown in table 3. One half of the patients have died as a direct result of brain metastasis, or have failed treatment to the brain and are still alive after re-treatment. Five of the 18 patients who died from brain metastases had no other clinical evidence of SCCL, ie, their extracranial metastases had responded completely to chemotherapy. Of the 18 patients who died from extracranial metastases, five were autopsied and four had uncontrolled brain metastases. Two patients also had leptomeningeal spread of disease; another had spinal cord compression, but autopsy was not permitted.

One of five patients who were asymptomatic, but had positive radionuclide or CT scans at presentation, is free of disease. He received cranial and thoracic irradiation and systemic chemotherapy with cyclophosphamide, doxorubicin (Adriamycin), and vincristine, and is well 11 months after the diagnosis.

The only morbidity that could be attributed to therapeutic brain irradiation was epilation. This side effect would also be expected from the combination chemotherapy. Although late brain damage from irradiation and chemotherapy would be difficult to distinguish from recurrent tumor, there was no evidence of late neurologic deficit that stabilized. All late signs of intracranial disease were

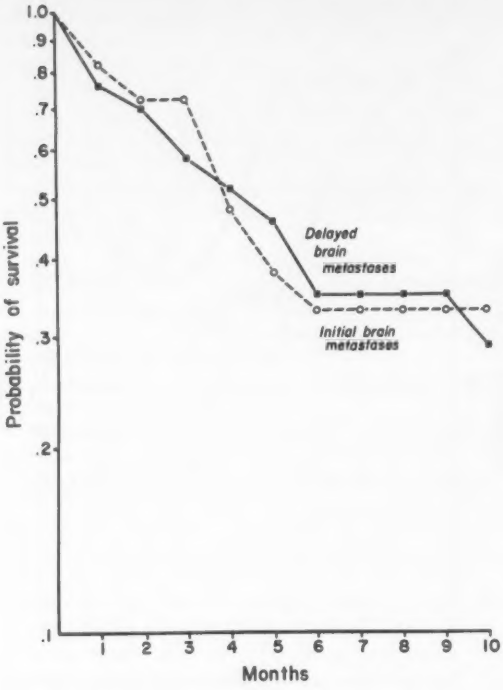

FIGURE 3.—Survival from diagnosis of brain metastases.

progressive and were attributed to failure to control the brain metastasis.

DISCUSSION

The brain is a frequent site of failure after treatment for SCCL. In one reported chemotherapy series (5) eight of 51 (15%) patients had failed treatment because of disease in the brain. Another compilation of chemotherapy series showed 73 of 325 (22%) patients with progressive disease in the brain (2). Sixty-six of the 165 patients in the current series who did not have brain metastasis at the time of diagnosis received prophylactic cranial irradiation; thus, clinical intracranial failure was seen in 17 of 99 (17%) of these patients. Results from the last MCWAH study that did not utilize prophylactic brain irradiation (6) showed eight of 27 (30%) patients with intracranial metastasis (median survival of 48 weeks).

In studies from the Veterans Administration Lung Group, 37 of 82 (45%) patients with SCCL had brain metastasis at autopsy (7). A review of

TABLE 3.—Current status of patients with brain metastasis from SCCL

Status	No. of patients
Dead from brain metastasis	18
Dead from extracranial metastasis	18
Alive with disease	3*
Alive, no evidence of disease	1
Total	40

*2 patients had irradiation repeated for brain metastases.

studies reported between 1960 and 1975 showed that 223 of 567 (39%) patients had brain metastases at autopsy; studies reported in 1977 and 1978 found this rate to be 50% (86 of 173 patients) (8). Continued improvement in the survival of patients with SCCL due to disease control outside the brain with systemic chemotherapy could result in a clinical failure rate in the brain close to the 40%–45% rate found at autopsy. Nugent et al (9) found that the combined, ie, clinical and autopsy, intracranial failure rate reached 80% for patients who survived > 24 months.

It is apparent that irradiation of the brain after the clinical appearance of metastasis from SCCL will not be able to rival the benefits of prophylactic irradiation. Despite early recognition of brain metastasis, the disease can progress so rapidly that prompt intervention with steroids and irradiation is still too late. Evidence of this is provided by eight patients who failed to complete the planned course of treatment to the brain. To have excluded these patients because they did not complete treatment would have inappropriately minimized the threatening character of involvement at this site.

The Radiation Therapy Oncology Group and the Eastern Cooperative Oncology Group recently completed a randomized study of prophylactic brain irradiation in patients with small cell carcinoma limited to the lung and regional lymph nodes (10). All patients received immediate or delayed chemotherapy with cyclophosphamide and CCNU plus local-regional irradiation. The most recent data show that the different approaches to chemotherapy did not affect median survival or pattern of failure. However, three of 99 (3%) patients who received cranial irradiation with 3000 rads in ten treatments developed brain metastases compared with 18 of 101 (18%) patients who had no brain irradiation ($P < 0.01$). A review of the available data from controlled and uncontrolled series showed that approximately 5% of patients who receive prophylactic brain irradiation subsequently manifest cerebral metastases (3). In the absence of such prophylaxis, the present study indicates that therapeutic brain

irradiation can control the intracranial disease in one half of the patients who develop brain metastasis. Assuming that the risk of brain metastasis is 50% for patients who survive at least 1 year (9), nearly one quarter of all patients with small cell carcinoma might benefit from irradiation for subclinical disease. However, long-term followup of patients with prophylactic brain irradiation may also show a higher intracranial failure rate than has been recognized to date.

In conclusion, therapeutic brain irradiation is not sufficiently effective, nor can it predictably be offered with the immediacy necessary after development of neurologic symptoms and signs, to obviate the need for prophylactic brain irradiation in patients with SCCL. As chemotherapy regimens become more effective and long-term survival improves, prophylactic irradiation of the brain, and perhaps other sanctuary sites, will assume greater importance.

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Augmentation of Vincristine Neurotoxicity by Irradiation of Peripheral Nerves[1]

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SUMMARY

Four cases are presented in which children receiving radiation therapy and combination chemotherapy had unilateral severe and prolonged neurotoxic effects. Augmentation of vincristine neurotoxicity by irradiation of peripheral nerves is suggested.

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Unanticipated interactions between radiation therapy and a variety of chemotherapeutic agents have been described. Vincristine is known to produce a peripheral neuropathy which is often of clinical importance, and may produce autonomic and central neurotoxicity as well (1,2). The mechanism of such toxicity remains in question (3), but it is known to be strongly dose-dependent (4) and can be dose-limiting in clinical application. Regional radiation therapy is often used in conjunction with vincristine chemotherapy in the treatment of various childhood tumors and lymphomas. On the basis of our observations of four patients we suggest that there may be an accentuation of severity and persistence of vincristine neurotoxicity by irradiation of peripheral nerves.

CASE REPORTS

Patient 1

Patient 1 is a 13-year-old male with a 5-month history of right groin swelling. Aspiration biopsy of a right femoral lymph node revealed synovial cell sarcoma. Upon referral, an exophytic lesion of the right second toe, which proved to be the primary, was noted and was treated by amputation.

Radiation therapy consisted of 4525 rads in 37 days to the paraaortic and right iliac lymph nodes and 5525 rads in 44 days to the right inguinal and femoral region, delivered by 4-MeV linear accelerator. An additional 674 rads in 2 days was given to the inguinal and femoral region using 7-MeV electrons. The patient then received doxorubicin (45 mg/m^2 every 3 weeks) and DTIC (150 mg/m^2/day for 5 days every 3 weeks) for 5 months, and after a cumulative doxorubicin dose of 450 mg/m^2 his chemotherapy was changed to vincristine (2 mg/m^2/week for 7 weeks, then every 3 weeks), actinomycin D (1.25 mg/m^2 every 3 weeks), and cyclophosphamide (750 mg/m^2 every 3 weeks) (VAC). Within 2 months of starting VAC therapy, bilateral loss of deep-tendon reflexes, hoarseness, and weakness of dorsiflexion of the right foot were noted. Ten months later severe right foot drop was present and vincristine was discontinued. Within 7 months only weakness of dorsiflexion of the right foot remained, and at 20 months after discontinuing vincristine full strength had returned. The patient remains continuously tumor-free for 44 months, but retains a minor paresthesia of the right foot.

Patient 2

Patient 2 is a 14-year-old female with a 2-month history of right lower leg pain who was found to have a diffuse lymphoblastic lymphoma of the tibia without evidence of disease elsewhere.

She was treated with 4-MeV X-rays; 4000 rads in 28 days to the entire right tibia and 5600 rads in 40 days to the area of soft tissue swelling and radiographic abnormality. Concurrent treatment was begun with doxorubicin (45 mg/m^2 every 3 weeks

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to a total of 450 mg/m², prednisone (40 mg/m²/day for 21 days, then 120 mg/m²/day for 5 days every 3 weeks), and vincristine (2 mg/m²/week for 4 weeks, then every 3 weeks). Four weeks after the institution of therapy the vincristine dose was reduced to 1 mg/m² because of neurotoxicity consisting of constipation and tingling of the fingers and toes. Eleven months thereafter bilateral ankle weakness of dorsiflexion was noted, which progressed in the right foot during the ensuing 6 months. All chemotherapy was electively stopped after a total of 22 months of treatment. At that time severe right and mild left foot drop were noted, and several episodes of "buckling" of the right leg had occurred. Shortly after stopping treatment, one such "buckling" resulted in a fracture of the tibia and fibula. One year later only mild right-sided weakness of dorsiflexion persisted, and the fractures had healed. The patient remains continuously tumor-free for 38 months.

Patient 3

Patient 3 is a 9-year-old female with a 3-month history of a nontender firm tumor of the left orbit. A biopsy revealed embryonal rhabdomyosarcoma and no evidence of metastatic disease could be demonstrated.

She received 5580 rads in 47 days via 4-MeV X-rays to the left orbit and neck nodes. Concurrent VAC chemotherapy was administered as in patient 1. Eight weeks after starting therapy, bilateral ptosis and mild loss of deep-tendon reflexes were noted. Seven months thereafter a marked increase in the left-sided ptosis and a decrease in the motility of extraocular muscles of the left eye were present. Vincristine was discontinued and within 8 months only a persistent, slowly resolving left ptosis was present. She remains continuously tumor-free for 21 months, and her sight in the affected eye is good.

Patient 4

Patient 4 is a 14-year-old male with pain and swelling of the left calf who was found to have a tumor of the fibula with considerable extension into soft tissues. A biopsy of the soft tissue tumor revealed Ewing's sarcoma.

Treatment was begun with VAC chemotherapy as in patients 1 and 3. Concurrently, radiation therapy was begun, delivering 4000 rads in 26 days to the entire fibula and 5800 rads in 41 days to the area of the tumor. Three weeks after completing radiotherapy he had symmetric strength but bilaterally diminished ankle jerks, and the tumor had disappeared. At 5 weeks he had weakness of dorsiflexion on the left, and at 7 weeks he had slight weakness on the right with a moderately severe foot drop on the left. This finding persists with slight progression on continuing chemotherapy.

DISCUSSION

Damage to peripheral nerves, particularly the brachial plexus, is occasionally observed as a result of radiation therapy, but generally only at high total doses or with large daily fractions (5). The neurotoxicity produced by vincristine is usually symmetric and distal, although ocular palsies and unilateral facial weakness have been described (6). Byfield (7) described an infant who developed radiation myelitis of the cervical spinal cord after receiving routine doses of vincristine and radiation therapy, and postulated a synergistic effect. Watkins and Griffin (8) reported a higher incidence of peripheral neurotoxicity due to vincristine in patients with lymphoma than with other tumors (mostly breast carcinoma), and it is tempting to postulate that patients with lymphoma were more often treated with metachronous radiation therapy. However, the authors give no information regarding radiation treatment and there is no mention of unusual or asymmetric neurotoxic effects.

The patients described in this report differ from each other in age, site of primary tumor, histologic diagnosis, and chemotherapeutic regimen received. None, however, manifested neuropathy attributable to his tumor, and none had a radical surgical procedure or biopsy in a site which might produce subsequent neuropathy. Bilateral neurotoxic effects occurred in all four children, but in each case they were clearly more severe on the affected, irradiated side of the body. Vincristine toxicity is clearly indicated by the time of onset of neuropathy and the variable resolution of toxic signs and symptoms following discontinuation of the drug. Local neuropathy has persisted only in areas where the pertinent peripheral nerves have been irradiated. We are unaware of any irradiated patients who demonstrate asymmetric vincristine toxic reactions in whom the *un*irradiated side is more severely involved; this suggests an interaction between vincristine and radiation therapy.

The mechanism whereby radiation might augment the peripheral neurotoxicity of vincristine is not known. It has been suggested that vincristine administration may make peripheral nerves more susceptible to physical trauma, and that vincristine may delay recovery of nerves injured through entrapment by tumor (9). Anecdotal reports of augmented vincristine toxicity in patients with pre-existing neurologic disorders such as Charcot-Marie-

Tooth disease or myotonic dystrophy have appeared. A closely observed patient with diabetic neuropathy, however, had neurotoxic effects no different from those of other patients who received similar drug doses. Available biopsy and necropsy material on human patients suggests axonal degeneration and neurofilament abnormalities as the mechanism responsible for vincristine neurotoxicity (10), in contrast to radiation neuropathy where demyelination is the usual anatomic lesion.

It is also unknown whether the interaction described herein is limited to children or merely requires a high dose of irradiation combined with repeated doses of vincristine, a setting more commonly seen in pediatric than adult oncology. We have not attempted a rigorous review of all patients seen at our institutions receiving unilateral radiation therapy and multiple courses of vincristine; we, therefore, cannot estimate the true incidence of this augmented toxicity. However, observation of the phenomenon in at least four patients suggests that it is not rare and may assume clinical importance.

Vincristine is generally used in combination with other chemotherapeutic agents, where a significant virtue is its relative lack of myelosuppression. In each of these cases vincristine was considered vital to the drug regimen employed and was continued as long as possible, even after the development of significant neuropathy. Optimal management in such cases is uncertain; substitution of vinblastine, which has its own neurotoxicity, might lead to unacceptable myelosuppression. In the final analysis the survival of the patient is paramount, and neuropathy may prove to be an unavoidable cost of treatment in certain cases.

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Peripheral Leukocytes as Indicators of the Enzymatic Effects of *N*-(Phosphonacetyl)-L-aspartic Acid (PALA) on Human L-Aspartate Transcarbamoylase (ATCase) Activity[1]

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SUMMARY

The interaction of *N*-(phosphonacetyl)-L-aspartic acid (PALA) with L-aspartate transcarbamoylase (ATCase), the putative target for the antineoplastic activity of this drug, has been studied in the blood of patients participating in a phase I trial of PALA. ATCase activity in human blood is most abundant in granulocytes and lymphocytes; comparatively little activity is seen in erythrocytes. Utilizing peripheral leukocytes from patients given infusions of PALA, we find that leukocyte ATCase is inhibited rapidly and strongly. After cessation of therapy the rate of restitution of enzyme activity is slow: half-maximal restoration is achieved in about 280 hours. As a correlate of this gradual recovery of enzymatic activity, nanomolar concentrations of PALA are detectable in the plasma 2 weeks after infusion. The apparent K_i of PALA for leukocyte ATCase with carbamoyl phosphate as the variable substrate is 5 nM. Uptake of PALA into leukocytes in vitro is saturable and occurs at a moderate rate comparable to that measured in murine tumor cells. Correspondingly, inhibitory concentrations of PALA ($\sim 10^{-7}$ M) are found in the leukocytes of patients throughout the course of PALA treatment. It is concluded that although leukocytes are not targets for PALA toxicity, they may serve as accessible and pertinent indicators of the enzymatic effects of this new oncolytic drug.

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N-(Phosphonacetyl)-L-aspartic acid (PALA) was synthesized as a stable analog of the transition-state intermediate of L-aspartic acid transcarbamoylase (ATCase), the second enzyme in the pyrimidine biosynthetic pathway (1). As such, PALA acts as a potent inhibitor of de novo pyrimidine biosynthesis in mammalian cells (2,3). PALA also exhibits striking antineoplastic activity against a broad spectrum of transplantable solid tumors, although it is ineffective against murine leukemias (4–7). The antitumor activity of PALA in vivo can be reversed by uridine, and more importantly, by carbamoyl-L-aspartic acid; this finding strongly suggests that oncolysis is achieved through the starvation of cells for this pyrimidine precursor (4). Both in vivo and in vitro, the sensitivity and resistance of murine tumors to PALA appear to correlate with low and high activities of ATCase, respectively (5–8). While this enzymatic target for the antineoplastic activity of PALA has been extensively studied in mice, little is known about the effect of PALA on ATCase in man. For this reason, we studied the kinetics of inhibition of ATCase by PALA in the blood of patients participating in a phase I trial with the drug,

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and we assessed the utility of peripheral leukocytes as indicators of the enzymatic effects of PALA.

MATERIALS AND METHODS

Chemicals.—L-[U-^{14}C]aspartic acid (216 millicuries/mmol) and L-[4-^{14}C]aspartic acid (19.6 millicuries/mmol) were purchased from Amersham/Searle Corp, Arlington Heights, IL. The dilithium salt of carbamoyl phosphate and L-glutamate oxaloacetate transaminase (EC 2.6.1.1), 10 mg/ml (180 IU/mg of protein), were obtained from Boehringer Mannheim Ltd, Elmsford, NY. Pyruvate decarboxylase (EC 4.1.1.1), 12 mg/ml (18 IU/mg of protein), was a product of Sigma Chemical Corp, St. Louis, MO. Versilube-F50 silicone oil was obtained from the Harwick Chemical Corp, Cambridge, MA. Acetyl-[^{14}C]PALA (23 millicuries/mmol) was obtained through the National Cancer Institute, Bethesda, MD, and was chromatographed on a 2 × 40-cm DEAE Sephadex A-25 column eluted with a 0–6-M formic acid gradient. Radiochemical purity of 98% was confirmed by analysis on a Jeol amino acid analyzer, JLC-5AH, equipped with a 1 × 10-cm column packed with Hamilton HA-x4 resin and eluted with a series of lithium citrate buffers (9). All other reagents were commercially available and of analytic quality.

Leukocytes.—A buffy coat was isolated from heparinized whole blood following low-speed centrifugation (150 × *g*, 5 minutes). Contaminating erythrocytes were removed by brief hypotonic shock and recentrifugation (10). Typically, 5 ml of blood was drawn from patients undergoing PALA therapy, heparinized, and subsequently fractionated to yield plasma for determination of PALA concentration and leukocytes for ATCase assay. Leukocytes used to study ATCase kinetic parameters and in vitro PALA uptake were obtained from healthy volunteers and were prepared in the same manner but on a larger scale.

Measurement of ATCase.—Leukocytes were suspended in a minimal volume (generally 200 µl) of buffer containing 30% DMSO, 5% glycerin, 0.1 M Tris-HCl (pH 8.4), 0.1 M KCl, 0.2 mM EDTA, and 2 mM dithiothreitol. Following cell rupture by repeated (3 ×) freeze-thawing, the homogenate was spun at 12,000 × *g* for 3 minutes in an Eppendorf centrifuge. The supernatant was assayed for ATCase activity as described (8), except that the 37°C incubation was for 60 minutes. Protein determinations were made with the Bio-Rad dye-binding reagent (Bio-Rad Laboratories, Richmond, CA) (11). ATCase activity was expressed as nanomoles of carbamoyl-L-aspartic acid formed per milligram of protein per hour. For kinetic studies the reaction mixture (in a total volume of 20 µl) contained either 5 µl of L-[4-^{14}C]aspartic acid (final concentration: 0.078–5.0 mM) and 5 µl of 4 mM carbamoyl phosphate *or* 5 µl of L-[U-^{14}C]aspartic acid and 5 µl of carbamoyl phosphate (final concentration: 1.25–10 µM in 0.05 M Tris-HCl, pH 8.4). Additionally, vessels also contained either 5 µl of PALA (final concentration: 0.01, 0.04, or 0.4 µM) or 5 µl Tris-HCl, pH 8.4, and 5 µl of leukocyte extract. The reactants were mixed by brief centrifugation at 12,000 × *g* and were incubated at 37°C for 10 minutes. The reaction was terminated by heating at 95°C for 2 minutes. Dissipation of the unreacted radiolabeled L-aspartic acid was accomplished enzymatically as described elsewhere for L-[4-^{14}C]aspartic acid (8) and L-[U-^{14}C]aspartic acid (12). Reaction vessels were then placed in a toluene-based scintillation fluid and counted at 85% efficiency in a Beckman LS-230 scintillation spectrometer.

Estimation of PALA.—The concentration of PALA in the plasma and leukocytes of patients participating in the phase I trial was determined by radiometric enzyme-inhibition assay (13). For plasma samples drawn at later time points after PALA treatment, where greater sensitivity was required, the modification of Kensler et al (14) was used. Plasma samples were diluted and deproteinized prior to assay as described (14).

Transport of PALA.—Leukocytes were suspended at a concentration of 1 × 10^6 cells/ml of Dulbecco's phosphate-buffered saline plus 5.6 mM glucose. Transport studies were performed at 37°C with gentle agitation of the cells. [^{14}C]PALA was added to leukocyte suspensions at the concentrations indicated: at appropriate times, 900-µl aliquots of cells were transferred to microcentrifuge tubes containing 600 µl of Versilube-F50 silicone oil. To terminate transport, cells were spun through the oil at 12,000 × *g* for 1 minute in an Eppendorf centrifuge. The cell pellets were solubilized in 1.5 ml of 0.2 N NaOH at 37°C for 3 hours. Samples were then neutralized and suspended in 15 ml of Aquasol (New England Nuclear Corp, Boston, MA) prior to scintillation counting. Data were corrected for entrapped extracellular drug by subtracting the radioactivity measured in cells following a 1-minute incubation in the presence of the appropriate [^{14}C]PALA concentration.

RESULTS

The distribution of ATCase activity in the elements of human blood is shown in table 1. Enzyme activity is lowest in the erythrocyte fraction and highest in the leukocyte fraction; in the ten individuals examined the leukocyte ATCase activity is

TABLE 1.—Distribution of ATCase in human blood*

Blood fraction	ATCase activity†
Whole blood	2
Erythrocytes	7
Leukocytes	305
Granulocytes	318
Lymphocytes	356
Plasma	0.1
Platelets	253

*Heparinized whole blood from a normal volunteer was fractionated into erythrocyte, leukocyte, and plasma components by centrifugation at 150 × *g* for 5 mins. Granulocytes and lymphocytes were prepared by standard Ficoll-Hypaque sedimentation of leukocytes. Platelets were isolated by centrifuging plasma at 12,000 × *g* for 5 mins. Cells were disrupted by freeze-thawing and ATCase was assayed in the 12,000 × *g* supernatant as described in the Materials and Methods section.

†nmols of carbamoyl-L-aspartate formed/mg of protein/hr.

266 ± 33 (mean ± SE) nmols of carbamoyl-L-aspartate formed/mg of protein/hour. Granulocytes and lymphocytes have approximately equal specific activities, but because of relative cell numbers, granulocytes are the major source of leukocytic ATCase activity. On the basis of this distribution and because leukocytes are the nucleated cells most easily obtained in sufficient numbers for experiments in vitro, it was decided to examine human peripheral leukocytes as indicators of the enzymatic effects of PALA.

Patients involved in the phase I trial of PALA at the National Cancer Institute received five daily infusions (15 minutes) of PALA on a 3-week cycle. Figure 1 shows the effects of five daily 1500-mg/m² (33-mg/kg) infusions on one patient's peripheral leukocyte ATCase activity during the first course of PALA therapy. Although basal leukocyte ATCase activity in this patient is the highest of all individuals examined (range, 165–480 nmols/mg of protein/hour), the marked inhibition (~ 80%) immediately following the first infusion of PALA and throughout the course of treatment is typical of the response observed. Inhibition of the enzyme is persistent; after cessation of therapy 280 hours are required to achieve half-maximal restitution of activity. Similar protracted rates of restitution of ATCase are also seen in rodent tumors and tissues after treatment with single doses of PALA (8,15).

The time course for the removal of PALA from the plasma of this patient is shown in figure 2. After

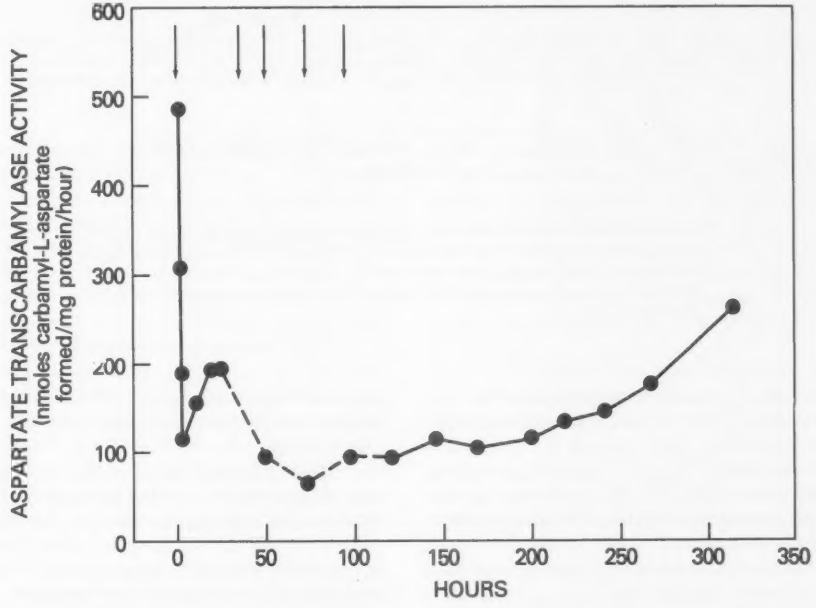

FIGURE 1.—Time course of the inhibition of leukocyte ATCase by PALA. The patient received 5 daily 15-min, 1500-mg/m² infusions of PALA at the indicated times (▾). Just prior to each PALA infusion and at the other time points, 5 ml of heparinized blood was drawn and leukocyte ATCase was assayed as described in the Materials and Methods section. Points = means of quadruplicate determinations.

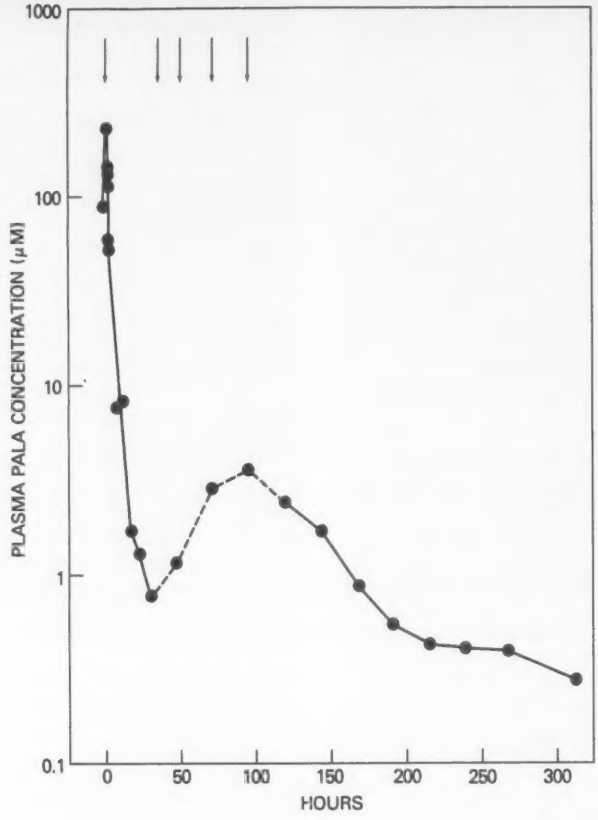

FIGURE 2.—Time course of removal of PALA from the plasma of the patient described in fig 1. PALA concentrations in the plasma of the samples obtained for leukocyte ATCase assay were determined using the enzyme-inhibition assay described in the Materials and Methods section. Points = means of triplicate determinations.

attaining an initial concentration of about 200 μM, plasma levels drop 100-fold during the 24 hours following PALA infusion. The half-life for this phase of elimination is about 3 hours, in accord with values reported elsewhere (16,17). In addition to this phase of rapid clearance of PALA following the first and subsequent infusions, there is also a protracted secondary elimination phase with an approximate half-life of 50 hours. On the basis of this slow half-life, it can be predicted that PALA plasma levels should be about 10 nM at 16 days after the last infusion. In fact, in four patient samples assayed at this time point, a 10–30-nM range of PALA plasma concentrations has been observed. Correspondingly, at this same time, ATCase activities in the leukocytes of these patients were still inhibited by about 20%.

A further indication of whether these persistent low plasma levels of PALA might be of potential therapeutic importance can be gleaned from a kinetic analysis of ATCase in leukocytes. As shown in figure 3, PALA is a competitive inhibitor with carbamoyl phosphate as the variable substrate but is noncompetitive with respect to L-aspartic acid. The apparent K_i versus carbamoyl phosphate is about 5 nM, indicating the potent activity of this drug. The apparent K_ms of leukocyte ATCase for carbamoyl phosphate and L-aspartic acid are about

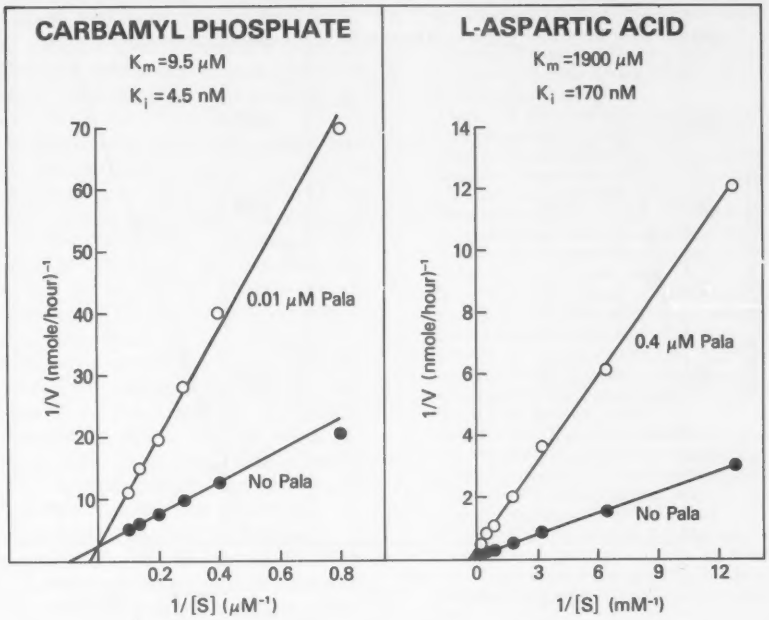

FIGURE 3.—Kinetic studies on the inhibition of leukocyte ATCase by PALA, with carbamoyl phosphate or L-aspartic acid as the variable substrate. Studies were conducted as described in the Materials and Methods section. Points = means of triplicate determinations.

10 and 1900 μM, respectively. These values are similar to those reported for both mouse spleen and murine tumors (8,18). Clearly then, levels of PALA in the plasma persist for several weeks in excess of those theoretically required to inhibit leukocyte ATCase by 50%.

It becomes important, then, to determine the intracellular concentrations of PALA during the course of therapy. Approximate calculations, based on measuring intracellular PALA on a per-milligram-of-protein basis and then assuming that 1 mg of protein is equivalent to 10-μl intracellular volume (ie, a cell is ~ 10% protein by weight [19]) suggest that levels of about 10^{-6}–10^{-7} M PALA are achieved in patient leukocytes immediately following the cessation of therapy. In order to study PALA transport more directly, peripheral leukocytes suspended in Dulbecco's phosphate-buffered saline plus glucose were incubated at 37°C in the presence of concentrations of [^{14}C]PALA (10^{-4}–10^{-6} M) equivalent to those seen in vivo immediately following PALA infusion. Uptake of the drug is moderately rapid. After a 3-hour incubation with 100 μM [^{14}C]PALA, leukocytes accumulate about 30 pmols of [^{14}C]PALA/10^6 cells, a figure roughly equivalent to an intracellular concentration of 30 μM. [^{14}C]PALA uptake is linear with time throughout this period. Using similar incubation conditions, our laboratory has previously shown that murine tumor cells in vitro accumulate between 10 and 50 pmols PALA/10^6 cells in 3 hours (8). Kinetic studies shown in figure 4 indicate that PALA uptake by leukocytes is saturable and has an apparent K_m of 0.4 mM. Leukocytes which are preincubated in the presence of 2 mM L-aspartic acid for 30 minutes and then concurrently exposed to 200 μM [^{14}C]PALA for 2 hours show a reduction in PALA uptake of about 90%, suggesting that these two compounds may share a common transport process. Overall, these results suggest that uptake of drug into leukocytes is not a limiting factor for the expression of PALA activity, either in vitro or in vivo.

DISCUSSION

Pharmacokinetic studies of PALA in mice show the drug to be present in plasma at nanomolar concentrations for up to 1 month following single-dose

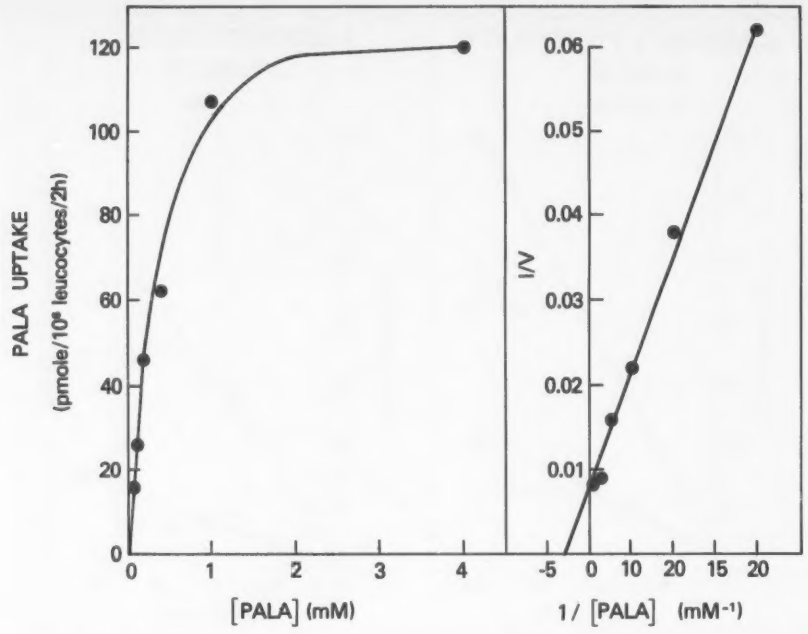

FIGURE 4.—Transport of PALA by leukocytes in vitro. Leukocytes were incubated at 37°C for 2 hrs in the presence of 0.1–4 mM [^{14}C]PALA. Accumulation of the drug was measured according to the procedure detailed in the Materials and Methods section. Points = means of quadruplicate determinations.

administration (14). Using whole-body radioautography, Chadwick et al (20) have shown that bone is the primary site of long-term PALA accumulation. Direct measurement of PALA levels in mouse femurs 1 month after a single ip dose of 400 mg/kg reveals a PALA concentration of ~ 10^{-4} M (B. Ardalan, unpublished observation), presumably reflective of the general avidity of phosphonates for bone. The likelihood of a similar long-term retention of PALA in man is strongly suggested by the very protracted persistence of PALA in the plasma of the patients examined in this study.

Hoogenraad (18) has described the apparent K_i of PALA towards ATCase from mouse spleen to be 26 nM. This fact, coupled with the observation that a single dose of PALA can inhibit splenic ATCase for 2 weeks (15), suggested that the long-term presence of PALA in the plasma of mice could be of pharmacologic importance. Concordantly, in the present studies we find that human leukocyte ATCase with an apparent K_i of 5 nM with respect to PALA is persistently inhibited, with 11 days required to achieve half-maximal restitution of enzyme activity. In this context, it is not surprising that the toxicity and therapeutic effects of PALA are not prominently schedule-dependent (16, 17,21–23).

The specific activity of ATCase is known to be an important determinant of tissue and tumor sensitivity to PALA (5,8). Human leukocytes, with a high ATCase activity of about 300 nmols of carbamoyl-L-aspartate formed/mg of protein/hour, are not likely to be targets for PALA toxicity; the same is true of their progenitors inasmuch as little myelosuppression is seen in patients treated intensively with the drug. Animal studies corroborate that PALA induces little hematotoxicity (5). Human skin and colon mucosae, by contrast, are sites of dose-limiting toxic effects (16,17,21–23). As might be expected, we have found ATCase activity to be more than five times lower in these latter two tissues than in leukocytes (24).

Transport of PALA into human leukocytes is quite rapid. Intracellular concentrations well in excess of those theoretically required to half-maximally inhibit ATCase are readily achieved within

a 1-hour exposure to PALA at a concentration of 100 μM. We observe that inhibition of ATCase in vivo is maximal within 1 hour following PALA infusion. The relative insensitivity of leukocytes to PALA is therefore not a function of poor drug uptake.

The maximal inhibition measured rarely exceeded 80%–90% but may be artifactually low due to (*a*) dilution of the drug in the preparation of homogenates for the enzyme assay and (*b*) use of high concentrations of the competitive substrate, carbamoyl phosphate, in the assay. The extent of inhibition of ATCase in situ could thus be much greater than indicated by this assay. Nevertheless, ATCase is present in considerable excess over other enzymes in the de novo pyrimidine nucleotide biosynthetic pathway. In human lymphocytes, the rate-limiting enzyme in this pathway, carbamoyl phosphate synthetase II, has a 100-fold lower activity than ATCase (25). Thus, even a 99% inhibition of leukocyte ATCase might not materially impede precursor flux through this pathway.

Although human leukocytes are not targets for PALA toxicity, overall they do serve as accessible and pertinent indicators of the enzymatic effects of this new oncolytic drug. Transport studies suggest that leukocytes achieve intracellular PALA concentrations that approach those of plasma and that are many times greater than the K_i of the drug for its enzymatic target. Enzymologic studies, both in vivo and in vitro, suggest that human ATCase is sensitive to inhibition by PALA in a manner analogous to that observed in other mammalian species.

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In Vitro and In Vivo Preclinical Chemotherapy Studies of Human Neuroblastoma[1,2]

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SUMMARY

Two human neuroblastoma cell lines, LA-N-1 and SK-N-MC growing in vitro and as subcutaneous tumors in athymic nude mice, were evaluated for their sensitivity to cyclophosphamide, doxorubicin (Adriamycin), and vincristine. In vitro, cyclophosphamide, following liver S-9 metabolic activation, and vincristine were significantly more cytotoxic to SK-N-MC than to LA-N-1 cells; doxorubicin was equally cytotoxic to both cell types. Treatment of nude mice bearing LA-N-1 and SK-N-MC tumors with cyclophosphamide and vincristine produced significant reduction (> 50%) in SK-N-MC tumor weights but not in LA-N-1 tumor weights. Doxorubicin failed to produce significant reduction in the weight of either the LA-N-1 or the SK-N-MC tumor. These sensitivities were generally similar to the clinical response of the tumors to these same agents. Such an in vitro and in vivo system using these and other neuroblastoma cell lines may provide a preclinical model for evaluating the activity of chemotherapeutic agents against human neuroblastoma.

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Neuroblastoma is the second most common malignant tumor of childhood. Most patients have metastatic disease at the time of diagnosis and, in general, the prognosis of such patients is poor. Clearly, new therapeutic approaches must be developed and tested.

Until recently, the only in vivo animal model system for testing chemotherapeutic agents against neuroblastoma was the C1300 murine neuroblastoma cell line (1); furthermore, there has been no quantitative in vitro test system for neuroblastoma. A number of human neuroblastoma cell lines are now available with morphologic and neurochemical properties similar to those of the tumors from which they were derived (2–8). These human cell lines may provide relevant models for in vitro and in vivo preclinical investigations aimed at developing improved treatment regimens.

In this investigation, we determined the sensitivities of two well-characterized human neuroblastoma cell lines, LA-N-1 (6) and SK-N-MC (2,6), to cyclophosphamide, vincristine, and doxorubicin (Adriamycin) in vitro and in athymic nude mice. Both patients from whom the cell lines were derived received all three agents. The patient from whom the LA-N-1 cells were derived showed no response to the three drugs, whereas the patient from whom

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the SK-N-MC cells were obtained responded initially to radiotherapy, cyclophosphamide and vincristine, and only minimally to doxorubicin. In general, there was a correlation between the drug sensitivities of the tumors in the patients and those of the cell lines in culture and in nude mice. Therefore, although the data are preliminary, we believe in vitro and in vivo model systems which employ these and other well-characterized neuroblastoma cell lines may be useful for assessing chemotherapeutic agents potentially active against human neuroblastoma.

MATERIALS AND METHODS

Cytotoxicity Assay

Log-phase cells were plated in tissue culture dishes (diameter, 60 mm) at concentrations of 10^4 LA-N-1 cells/5 ml or 2.5×10^3 SK-N-MC cells/5 ml of complete medium. Twenty-four hours later, vincristine or doxorubicin was added for a given exposure time. The drug was then removed, cells were rinsed with 2.5 ml of complete medium, and 5 ml of complete medium was added. Two weeks later, the medium was removed and dishes were dried overnight. Colonies were stained with methylene blue for 30 minutes, rinsed with water, and dried. The colonies were then counted using an automatic colony counter. Cytotoxicity was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{number of colonies in control dishes} - \text{number of colonies in treated dishes}}{\text{number of colonies in control dishes}} \times 100.$$

Plating efficiency was low for both cell lines, ie, approximately 1.5% and 3.0% for LA-N-1 and SK-N-MC cells, respectively. Cells were plated to yield approximately 100 colonies/control dish. Colonies scored were a minimum of 1 mm in diameter and there was no difference in the size of colonies scored between control and treated cultures. Statistical evaluation was performed on the results using the T test for unequal sample sizes (9).

Metabolic Activation

In vitro, cyclophosphamide requires metabolic activation primarily through microsomal enzymes in the liver. Consequently, a rat liver preparation was used for this purpose. Sterile homogenates of liver were prepared from Sprague-Dawley rats 5 days after they were injected ip with 500 mg/kg of Aroclor 1254 (Analab Inc, North Haven, CT). The homogenate was centrifuged at 9000 × *g* for 10 minutes and the resulting supernatant fraction (S-9) was stored in 0.5-ml aliquots in sterile plastic tubes at –70°C. The complete S-9 system was prepared similarly to the procedure described by Natarajan et al (10). To activate cyclophosphamide, 0.1 ml of the drug was added to 0.5 ml of S-9 to make a final cyclophosphamide concentration of 5 or 10 μg/ml. Approximately 1×10^6 cells in 0.1 ml were then added to each tube. The tubes were capped and incubated at 37°C in a shaker bath. The cells were then washed twice with complete medium and plated in the tissue culture dishes at the previously described concentrations. After 24 hours of incubation, the medium was changed. Fourteen to 21 days later the colonies were stained and counted as described above. Controls for these experiments were cells treated with S-9 alone.

Tumor Growth

SK-N-MC and LA-N-1 cells were grown to subconfluence in tissue culture flasks, removed with 0.25% trypsin-EDTA (Flow Laboratories, Rockville, MD), washed twice, and resuspended in complete tissue culture medium. SK-N-MC cells (10^7 cells, passages 69–136) or LA-N-1 cells (3×10^7 cells, passages 38–86) in 0.3–0.5 ml of complete medium were injected sc into the dorsum of each mouse. The mice were observed for the appearance of subcutaneous tumors (minimum diameter, 3 mm) and then tumor diameters were measured twice weekly with vernier calipers. Tumor weight was calculated by the following formula (11):

$$\text{weight (mg)} = \frac{\text{length} \times \text{width}^2}{2}.$$

The calculated weight was similar to the actual weight of the tumors when animals were killed.

Histologic examination was performed for tumors produced by each cell line. Both tumors were small cell malignant neoplasms consistent with neuroblastoma, and no gross or microscopic metastases were observed.

Chemotherapy In Vivo

Mice received cyclophosphamide (250 mg/kg), doxorubicin (6 mg/kg), or vincristine (2.0 or 1.75 mg/kg) ip when their tumors reached a calculated weight of 400–1500 mg. Nonlethal drug doses, which would produce an average animal weight loss of ≤ 10%, were chosen. Control animals received an equal volume of normal saline. Toxicity and drug effect were monitored by weighing the mice and measuring their tumors three times per week. Wbc

TABLE 1.—Cytotoxicity of chemotherapeutic agents for SK-N-MC and LA-N-1 cells in vitro

Agent	Concentration (μg/ml)	Exposure time (hrs)	% cytotoxicity* SK-N-MC	% cytotoxicity* LA-N-1
Vincristine	0.001	2	62 ± 18 (14)	8 ± 8 (7)
		24	100 (16)	53 ± 11 (14)
	0.075	2	100 (16)	56 + 11 (8)
		24	100 (16)	53 + 11 (12)
Doxorubicin	0.005	24	37 ± 15 (16)	33 ± 15 (15)
Cyclophosphamide†	5	1	85 ± 36 (10)	18 ± 16 (9)
	10	1	100 (10)	57 ± 22 (9)

*The No. of colonies in each treated dish was compared to the No. of colonies in control dishes at 2 wks following drug exposure, and the % cytotoxicity was calculated. Differences in cytotoxicity between SK-N-MC and LA-N-1 were statistically significant ($P < 0.001$) for vincristine and cyclophosphamide. Differences in cytotoxicity between SK-N-MC and LA-N-1 were not statistically significant ($P > 0.2$) for doxorubicin. Values are means ± SD. Nos. in parentheses = No. of dishes counted.

†With exogenous metabolic activation. Control dishes in these experiments were treated with S-9 only. 500 μg/ml of cyclophosphamide without added S-9 produced no cytotoxicity to the SK-N-MC cells even after 24 hrs of exposure. The cytotoxicity of the S-9 was negligible when compared to control cultures not exposed to S-9.

counts were obtained on Days 0, 4, and 7 after treatment. A significant tumor response was defined as a > 50% reduction in tumor weight from the start of treatment. Following a minimum observation period of 14 days, the mice were killed and representative animals from each group were autopsied.

RESULTS

Cytotoxic Effects of Chemotherapeutic Agents In Vitro

With our in vitro system we determined whether or not a differential cell-kill was produced by vincristine, cyclophosphamide, and doxorubicin in the SK-N-MC and LA-N-1 cell lines (table 1). Both vincristine and cyclophosphamide were significantly more cytotoxic for SK-N-MC than for LA-N-1 cells ($P < 0.001$). Exposure to vincristine (0.001 μg/ml) for 2 hours or to cyclophosphamide (5 μg/ml) for 1 hour killed 62% and 85% of the SK-N-MC cells, respectively, whereas the two drugs killed only 8% and 18% of the LA-N-1 cells, respectively, under the same conditions. In contrast, doxorubicin produced no differential cytotoxicity in the two cell lines.

Antitumor Effects of Chemotherapeutic Agents In Vivo

The responses of tumors produced by LA-N-1 and SK-N-MC cells in nude mice to cyclophosphamide and vincristine generally reflected their in vitro sensitivities (table 2). Cyclophosphamide induced a significant weight reduction in six of eight SK-N-MC tumors. Tumor regression began within the first week following treatment. The tumor disappeared completely in only one mouse. Regrowth of all SK-N-MC tumors began within 3 weeks following initial treatment. In contrast, the LA-N-1 tumors did not respond significantly to cyclophosphamide. Wbc counts dropped from 5000–10,000/mm³ to < 1000/mm³ by 4 days after treatment and returned to normal by 7 days.

TABLE 2.—Response of LA-N-1 and SK-N-MC tumors in nude mice to chemotherapeutic agents

Drug	Dose* (mg/kg)	Response† SK-N-MC	Response† LA-N-1
Cyclophosphamide	250	6/8	0/9
Vincristine	2	4/4	1/5
Vincristine	1.75	5/7	0/6
Doxorubicin	5	0/5	0/6

*Each agent was given as a single ip dose.

†No. of mice with a significant response (> 50% reduction in tumor weight)/No. of mice treated.

Vincristine also produced more tumor regression in mice with SK-N-MC tumors than in mice with LA-N-1 tumors. There was a 50% reduction in SK-N-MC tumor weight in all four mice treated with 2 mg/kg and in five of seven mice treated with 1.75 mg/kg. In contrast, only one of five LA-N-1 tumors responded significantly to 2 mg/kg and none of six responded to 1.75 mg/kg. Decrease in weight of SK-N-MC tumors was observed within the first week after treatment. However, as with cyclophospha-

mide, tumor regrowth began in all but one mouse within 3 weeks. Average animal weight loss for mice injected with 2 or 1.75 mg/kg of vincristine was 15% and 11%, respectively. No significant myelosuppression was observed.

Doxorubicin was not active against either tumor. None of five mice with SK-N-MC tumors and none of six with LA-N-1 tumors had significant tumor regression. Three animals died 6, 7, and 9 days after doxorubicin. At the time of death, neither the two SK-N-MC tumors nor the one LA-N-1 tumor showed any signs of weight decrease. Animal weight loss was moderate, ie, 8% and 3% in mice injected with SK-N-MC and LA-N-1 tumors, respectively. No significant myelosuppression was observed.

A group of mice with either LA-N-1 or SK-N-MC tumors were killed before and after treatment with cyclophosphamide to compare the histologic changes in a sensitive and resistant tumor. For SK-N-MC tumors, extensive necrosis was visible by 4 days after treatment. These changes proceeded to replacement by fibrotic areas with few viable tumor cells by 6–8 days. For LA-N-1 tumors, focal necrosis was visible by 4 days after treatment; this progressed to focal fibrosis with a slight decrease in the number of viable cells by 8 days.

DISCUSSION

The use of well-characterized human cell lines for in vitro and in vivo preclinical chemotherapy studies has several advantages. Rapid screening of multiple agents can be accomplished in tissue culture, and those drugs showing the greatest activity can then be examined in animal systems. Even agents requiring metabolic activation in vitro such as cyclophosphamide can be evaluated, and complex in vivo phenomena such as drug resistance can be studied more thoroughly in vitro. Furthermore, by using multiple human neuroblastoma cell lines with different biologic properties and chemotherapeutic sensitivities, the effectiveness of any single agent can be assessed against a heterogeneous group of tumors representing this malignancy.

The only previous preclinical chemotherapeutic studies of neuroblastoma have been performed in vivo using the C1300 murine model in syngeneic mice (1,12,13) or human neuroblastoma cells in nude mice (14). As single agents, BCNU, cyclophosphamide, and cytosine arabinoside were active against C1300 murine neuroblastoma; in combination, BCNU and cyclophosphamide were effective (1,12,13). Clinically, only cyclophosphamide among these agents has shown a marked effect against human neuroblastoma. Helson et al (14), treating tumors formed in nude mice by human neuroblastoma cell lines, obtained results similar to ours; SK-N-MC cells were sensitive to cyclophosphamide whereas SK-N-GO cells that were derived from a patient resistant to cyclophosphamide were also resistant to cyclophosphamide.

Other investigations have examined the sensitivity of different human tumors in nude mice to chemotherapeutic agents and generally have concluded that the response of the human tumor xenografts often closely reflects the human response. For example, a dramatic decrease in Burkitt's lymphomas growing in nude mice has been reported after treatment with cyclophosphamide (15). In addition, a pattern of drug sensitivity for a human malignant melanoma in nude mice has been found to be similar to the drug sensitivity of melanomas in man (16).

In our studies, one of the cell lines used (SK-N-MC) was sensitive to cyclophosphamide and vincristine in vitro and in the nude mouse. It was derived from a patient whose tumor had previously shown sensitivity to cyclophosphamide and vincristine (2). We cannot, however, determine from the clinical history whether the tumor in the patient retained this sensitivity at the time tissue was obtained for the establishment of the cell line since the patient had not received these specific agents for > 4 months. On the other hand, unequivocal doxorubicin resistance of the tumor in the patient was correlated with a similar result in the model system.

The second cell line (LA-N-1) was derived from a patient whose tumor was clearly resistant to cyclophosphamide, vincristine, and doxorubicin, and the response of the cell line in the in vitro and in vivo model system paralleled the response of the tumor to these drugs in the patient. Previous studies showed that the neurochemical and morphologic properties of these human neuroblastoma cell lines correlate well with those of the tumors from which they were derived (2,6,8).

These studies suggest that human neuroblastoma cell lines may provide a relevant model system for the clinical evaluation of conventional and new chemotherapeutic agents against human neuroblastoma. Further studies will be necessary to extend these initial results.

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Randomized Comparison of Two Combination Chemotherapy Regimens Containing Doxorubicin in Patients With Metastatic Breast Cancer: A Western Cancer Study Group Trial[1,2,3]

Brief Reports and Preliminary Communications

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SUMMARY

Ninety-six patients with metastatic breast cancer were entered in a prospectively randomized trial comparing a five-drug doxorubicin (Adriamycin)-containing regimen given in two different schedules. Both regimens included cyclophosphamide, methotrexate, 5-FU, prednisone, and doxorubicin. On one schedule, referred to as "combination" treatment, doxorubicin was given every 21 days and cyclophosphamide was given daily. On the less intensive "fixed-rotation" schedule, doxorubicin was given on alternative cycles every 42 days and cyclophosphamide was given for 21 days of the 42-day cycle. Response frequency and survival were comparable among patients receiving either regimen. Significantly less ($P < 0.05$) nausea and leukopenia occurred on the fixed-rotation schedule. Therefore, similar therapeutic benefit along with decreased toxicity was obtained by use of combination chemotherapy involving doxorubicin and cyclophosphamide given in the less intensive schedule.

[Cancer Treat Rep 64:981–984, 1980]

Advanced breast cancer is responsive to several chemotherapeutic agents, with doxorubicin (Adriamycin) appearing to be the most active single agent for this disease (1). Preliminary evidence suggested that the addition of doxorubicin to combination chemotherapy regimens could increase response frequency (2,3). In addition, studies comparing sequential single-agent regimens to concurrent combination regimens have demonstrated equivalent survival and decreased toxicity for the sequential approach in several investigations (4,5). Therefore, the purpose of this study was to determine whether the duration of response and survival of patients with metastatic breast cancer could be altered by changing the scheduling of administration of a five-drug combination chemotherapy regimen involving doxorubicin.

METHODS

Ninety-six patients with metastatic breast cancer were entered in the study. Eligibility criteria included: histologic diagnosis, no previous therapy with cytotoxic drugs, and measurable disease. Additive hormonal therapy was discontinued at least 3 weeks before entry. Pretreatment evaluation included the following: physical examination, cbc, chest roentgenogram, bone marrow examination, bone and liver scans, and electrocardiogram. Hematologic status was monitored weekly. Responses were classified as *complete*—the complete disappearance of all measurable lesions with the ap-

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[3]This study was reported in part in the Proceedings of the American Association of Cancer Research and ASCO 19:335, 1978.

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TABLE 1.—Treatment schedule for the fixed-rotation or combination chemotherapy regimens used in the treatment of metastatic breast cancer

Regimen*	Drug received on Day—					
	1	8	15	22	29	36
Combination						
Doxorubicin	X			X		
Methotrexate		X		X		X
5-FU	X		X		X	
Cyclophosphamide	XXXXXXXXXXXXXXXXXXXXXXXXXXX					
Fixed-rotation						
Doxorubicin	X					
Methotrexate	X	X	X			
5-FU				X	X	X
Cyclophosphamide				XXXXXXXXXXX		

*Doxorubicin, 5-FU, and methotrexate were given iv while cyclophosphamide and prednisone were given orally. Doses were as follows: doxorubicin, 30 mg/m²; 5-FU, 300 mg/m²; methotrexate, 20 mg/m²; cyclophosphamide, 100 mg; prednisone, 40 mg every other day from Day 1 to Day 42 then 20 mg every other day. Prednisone was given every other day in both treatment arms.

pearance of no new lesions for ≥ 1 month; or *partial*—a reduction in tumor of ≥ 50% of the cross-sectional area of all measurable lesions, with the appearance of no new lesions for ≥ 1 month. Minor change, no change, or progression of any measurable lesions during therapy was considered as no response.

Two schedules of chemotherapy were used (table 1). In both, prednisone was given every other day. On one schedule, referred to as "combination," the four remaining agents were given on a 21-day cycle. The second, less intensive schedule involved 50% less doxorubicin and cyclophosphamide and was referred to as "fixed rotation." On this schedule, therapy with doxorubicin and methotrexate was rotated with 5-FU and cyclophosphamide every 21 days, resulting in a 42-day cycle. Patients were randomly assigned to receive either combination or fixed-rotation regimens. In both treatment arms, doxorubicin was discontinued when a cumulative dose of 550 mg/m² was reached; the patients then continued to receive the remaining four drugs in the regimen. Patients with bone marrow involvement by tumor or a total bilirubin level of > 2 mg/100 ml initially began to receive doxorubicin at a dose of 20 mg/m². Dose modification was primarily determined by degree of bone marrow suppression at the time of drug administration.

RESULTS

Ninety-six patients were entered in the study. Four patients, all receiving the combination regimen, were lost to followup after receiving an initial dose of chemotherapy. One patient in each treatment arm was ineligible because of concurrent hormonal therapy. Therefore, 90 patients were evaluable for toxicity and response. The two treatment arms were comparable in regard to prognostic factors, prior therapy experience, and metastatic pattern (table 2).

Fifty percent of the combination group and 56% of the fixed-rotation group had an objective response. The response duration (period from achievement of response to the time of disease progression) also was similar in the two treatment arms

TABLE 2.—Pretreatment characteristics of patient groups

Regimen	Site of metastatic involvement*					Median age in yrs	Median Karnofsky performance status	Prior radiation therapy*	Previous hormonal therapy*
	Lung	Liver	CNS	Bone	Skin				
Combination	49	25	5	59	31	56	78	57	42
Fixed-rotation	48	22	6	58	50	55	80	63	38

*Values = % of patients.

(9.9 months for the combination group versus 8.6 months for the fixed-rotation group). The combination and the fixed-rotation groups experienced equivalent median survival (16.8 months for the combination group versus 18.4 months for the fixed-rotation group [difference not significant]). Responding patients had significantly longer median survival than patients with disease progression. Patients receiving the fixed-rotation regimen received a greater proportion of the scheduled chemotherapy than did those receiving the combination regimen. With the fixed-rotation regimen, 97% of the every-42-day doxorubicin dose could be given compared to 81% of the every-21-day dose in the combination regimen ($P < 0.05$). The difficulty in administering the scheduled chemotherapy in the combination regimen was associated with the more severe myelosuppression in patients receiving daily cyclophosphamide therapy. Thirty percent of the patients had initial bone marrow involvement. Nonetheless, the percentage of scheduled chemotherapy doses received was similar for patients with bone metastases, bone marrow involvement, and patients free of any bone disease. Survival was comparable when patients with bone metastases were compared to remaining patients.

Significantly less ($P < 0.05$) nausea and vomiting occurred in the fixed-rotation arm (33% versus 14%). Stomatitis occurred in 12% of the patients receiving either treatment. Leukopenia (wbc count $< 2000/mm^3$) was noted in 52% of the combination group and in 16% of the fixed-rotation group ($P < 0.05$). No correlation was seen between nadir wbc counts and response or survival in either treatment arm. Congestive heart failure (CHF) developed in association with doxorubicin treatment in two patients, both receiving the combination regimen. The first patient had fatal cardiomyopathy 34 months after receiving the last doxorubicin dose, at a cumulative level of 580 mg/m². The second patient had CHF 1 month after the last dose of doxorubicin, at a cumulative level of 425 mg/m². Doxorubicin-induced CHF was not observed in patients receiving the fixed-rotation regimen.

DISCUSSION

A combination regimen which included twice the dose of doxorubicin and cyclophosphamide did not increase response frequency or survival of patients with breast cancer, compared to a less intensive, fixed-rotation regimen. Although toxicity was significantly less in the fixed-rotation arm, neither regimen could be recommended for routine use since comparable survival has been obtained with less complicated, more convenient schedules (2,3).

The doxorubicin dose was 30 mg/m² in both regimens, lower than that used in many other trials. However, the optimal dose and schedule for doxorubicin administration in breast cancer remain to be determined. Low-dose doxorubicin (20 mg/m² on Days 1 and 8, every 28 days) was as effective in breast cancer as the standard dose (60 mg/m² every 21 days) in one randomized trial (6). In addition, two trials (7,8) of weekly doxorubicin administration have demonstrated response frequency of between 36% and 49% in patients with breast cancer. The doxorubicin dose interval was 42 days in the fixed-rotation arm in this study. Previously, single-agent doxorubicin was reported to have similar activity in breast cancer on a schedule of every 5 weeks compared to the usual schedule (9). Doxorubicin has been used as part of a combination in other breast cancer trials at intervals of 9 (10) and 6 weeks (11). Kennealey et al (11), in a trial involving a total of only 46 patients, observed a similar response frequency for a four-drug (doxorubicin, cyclophosphamide, methotrexate, and 5-FU) arm compared to a two-drug (doxorubicin and cyclophosphamide) arm with doxorubicin every 3 weeks. Therefore, less frequent doxorubicin administration in combination chemotherapy regimens can result in decreased toxicity with similar therapeutic benefit when compared to regimens involving doxorubicin given every 21 days.

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Treatment of Advanced Adult Acute Leukemia With Diglycoaldehyde: A Southwest Oncology Group Study[1,2]

Brief Reports and Preliminary Communications

Michael T. Shaw, Francis S. Morrison, W. J. Stuckey, and Arthur A. Trowbridge*

Diglycoaldehyde (DG) is a periodate-oxidation product of the purine nucleoside inosine, where the 2′ and 3′ carbons in the ribose moiety have been oxidized to formyl groups resulting in cleavage of the ring. DG has been shown to have modest activity in several animal tumor systems including AKR leukemia, Lewis lung carcinoma, and L1210 leukemia.

In a clinical phase I study 40 patients were treated by Kaufman and Mittelman (1) in dose schedules of 1.5 g/m² iv daily for 5 days every 10 days, 2 g/m² iv for 5 days every 10 days, or 3 g/m² iv for 5 days. Mild thrombocytopenia and leukopenia were observed in many patients with cell count nadirs at 10 days and complete recovery within 3–4 weeks after completion of a 5-day course. This hematopoietic depression was not seen until the dose was 2 g/m². In practice, the 2-g/m² doses were given for 3–5 days every 4 weeks instead of every 10 days to allow for hematologic and renal recovery. It is also interesting to note that the three responses occurred at this dose level. A Coombs' test-positive hemolytic anemia with a depression of 1–2 g/100 ml in the hemoglobin level after the first course was almost a constant finding. The anemia was not cumulative with repeated courses. Three to 5 days from the start of therapy the partial thromboplastin time became prolonged but returned to normal 5 days after the end of drug infusion; it was thought to be a drug-related effect in fibrinogen polymerization. The major dose-limiting toxic effect was renal tubular damage. At a dose level of 1.5 mg/m² daily for 5 days, patients had significant proteinuria by the fourth day which returned to normal within 2 weeks. This toxicity was not cumulative. All patients receiving unbuffered drug at a dose of 2 g/m² had evidence of severe localized venospasm and venous thrombosis at the site of infusion. Extravasation of the drug caused erythema, pain, necrosis, and swelling. These problems were overcome by buffering the solution to a pH of 7.4. Three patients with hypercalcemia due to osseous metastases from breast, renal, and squamous cell lung carcinomas became normocalcemic after receiving 2 g/m² of the drug daily for 3–5 days.

One complete response was achieved in a patient with seminoma. Two partial responses were achieved: one in a patient with malignant melanoma and one in a patient with oat cell carcinoma.

MATERIALS AND METHODS

Seventeen eligible adult patients, six males and 11 females, were entered in the study. All had advanced and drug-resistant acute leukemia. There were six patients with acute myeloblastic leukemia, four with acute myelomonocytic leukemia, one with acute monocytic leukemia, three with acute lymphocytic leukemia, two with acute undifferentiated leukemia, and one with chronic granulocytic leukemia in the blastic phase. The duration of disease in each patient varied from 6 to 258 weeks (median, 54 weeks). Patients were required to have a serum creatinine of ≤ 1.5 mg/100 ml and a BUN of ≤ 20 mg/100 ml. No patients with more than a trace of proteinuria were accepted.

DG was administered in five consecutive daily doses of 1.5 g/m² as a 6-hour iv infusion in 5% dextrose in water buffered to pH 7.4. Courses were repeated at 3-week intervals when possible. Bone marrow aspirations were done on Day 21. If the marrow was not hypoplastic, a further course was administered. No course was started before all evidence of proteinuria had disappeared, the BUN had

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TABLE 1.—Toxicity data

Toxic effect	No. of patients
Positive Coombs' test	3
Increasing creatinine and BUN	4
Proteinuria	2
Hypocalcemia	1
Diabetes insipidus	1
Postural hypotension	1
Nausea and vomiting	5

fallen to ≤ 20 mg/100 ml, and the serum creatinine was ≤ 1.5 mg/100 ml. Patients experiencing renal toxicity in the form of proteinuria of more than a trace or a BUN of > 20 mg/100 ml and a serum creatinine of > 1.5 mg/100 ml had their dose reduced by 25% in the subsequent course.

RESULTS

There were two early deaths. Of the 15 fully evaluable patients there were no complete remissions, and there was one partial remission in a patient with acute lymphocytic leukemia who had an M2 marrow for 3½ weeks and survived for 43 weeks following initiation of DG therapy. The survival time for all 17 patients ranged from 41 days to 43 weeks (median, 7 weeks). Two patients with acute myeloblastic leukemia are still alive at 28+ and 30+ weeks. Five patients received two courses of therapy, 11 received one course of therapy, and one received only 4 days of therapy because of early death.

Toxicity data are shown in table 1. Of interest is one patient who developed severe postural hypotension. Therapy had to be discontinued after the first course.

DISCUSSION

The results of this study, with only one partial response, are very disappointing. However, it must be remembered that these patients had far-advanced disease resistant to commonly used drugs. Similar disappointing studies have been noted by the Children's Cancer Study Group and by Boran and Hayat of France (F. Muggia, personal communication). The toxicity noted in our patients was similar to that described by others after use of this drug (1). The main complications were nausea, vomiting, renal toxicity, and a positive Coombs' test.

There is no justifiable reason that DG should be used in further phase II or phase III studies for the treatment of adult acute leukemia.

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Cause of Variability in Activity of Samples of CCNU Against P388 Leukemia In Vivo[1]

Brief Reports and Preliminary Communications

J. A. Montgomery[2,3,*]

CCNU was found to be highly active against L1210 leukemia (1) when it was used as the primary screen of the Division of Cancer Treatment. CCNU has been used at Southern Research Institute as a known positive control, and when the P388 leukemia became the primary screen in November 1975, it was evaluated in this animal system for use as a reference compound. Initially (data not shown) and later, it (Sample MH) gave a high percentage of cures, even against an inoculum of 10^7 cells, in the dose range of 50–75 mg/kg given on Day 1, 33 mg/kg being less effective and 100 mg/kg giving indications of chronic toxicity (*see* table 1). Samples of CCNU received later (ML-1 and NC-1) gave significantly fewer cures against both 10^6 and 10^7 cells and smaller increases in lifespan against 10^7 cells

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TABLE 1.—CCNU samples vs P388 leukemia in BDF_1 mice: ip treatment, Day 1

Sample	10^6 cells ip				10^7 cells ip			Toxicity controls, 60-day survivors§
	Dosage (mg/kg/dose)	60-day survivors*	Median day of death†	% ILS‡	60-day survivors*	Median day of death†	% ILS‡	
MH	33	2	24.5	104	0	18	100	9
	50	5	29	141	0	18	100	10
	60	9	28	133	6	28	211	9¶
	75	9	29	141	6	32	261	9
	100	9	20	66	4	24	172	10
ML-1	33	1	24	91	0	17	94	10
	50	0	22	79	0	14	55	10
	60	3	30	150	0	17	88	10
	75	2	30	150	0	17	88	10
	100	8	29	137	0	19	116	8
ML-2	33	0	25	83	0	15	66	10
	50	9	36	200	7	29	222	10
	60	10	—	—	9	38	322	10
	75	10	—	—	7	30	233	9
	100	4	20	62	6	26	183	5
NC-1	33	0	17	41	0	16	33	10
	50	1	21	75	0	17	38	10
	60	5	31	158	0	26.5	122	10
	75	6	28.5	137	2	22.5	88	10
	100	3	29	141	0	20	66	10
NC-2	33	5	33	175	2	26.5	122	9¶
	50	10	—	—	5	32	166	10
	60	8	29	145	5	32	166	10
	75	9	25	108	10	—	—	10
	100	6	13	8	9	50.5	322	7

*Of 10 animals.
†Of dying animals.
‡Of dying animals compared to untreated controls: median day of death = 12 for 10^6 cells, and 9 for 10^7 cells.
§Of 10 animals except where indicated. CMC control 10 of 10 60-day survivors.
¶Of 9 animals.

TABLE 2.—CCNU samples vs P388 leukemia in BDF_1 mice: oral and ip treatment, Day 1

Sample	Dosage (mg/kg/dose)	Route	10^6 cells ip			10^7 cells ip			Toxicity controls, 60-day survivors*
			60-day survivors*	Median day of death†	% ILS ‡	60-day survivors*	Median day of death†	% ILS ‡	
ML-1	0	—	—	—	—	—	—	—	10
	0	—	0§	11.0	—	0§	10.0	—	—
	100	Ip	4	20.0	81	1	16.0	60	10
	150	Ip	7	24.0	118	4	20.0	100	10
	40	Oral	0	14.0	27	0	12.5	25	10
	60	Oral	1	18.0	63	0	15.0	50	10
	90	Oral	3	20.0	81	1	17.0	70	9
	135	Oral	1	19.0	72	0	15.5	55	9
	200	Oral	2	22.0	100	3	20.0	100	8
ML-2	100	Ip	3	9.5	Toxic	2	11.0	Toxic	0
	150	Ip	0	7.0	Toxic	0	7.5	Toxic	0
	40	Oral	1	21.0	90	2	18.5	85	10
	60	Oral	7¶	19.5	77	4	24.0	140	10
	90	Oral	2	21.0	90	2	17.5	75	8
	135	Oral	0	14.5	31	0	13.0	30	0
	200	Oral	1	7.0	Toxic	0¶	7.0	Toxic	0

*Of 10 animals except where indicated. CMC control 10 of 10 60-day survivors.
†Of dying animals.
‡Of dying animals compared to untreated controls.
§Of 30 animals.
¶Of 9 animals.

at the same dose levels. An examination of the three samples by mass spectroscopy showed no differences, but sample MH was a finely divided solid, whereas ML and NC were both highly crystalline. Since all samples were administered as suspensions in CMC solution (*see* below), it seemed possible that the difference in physical state of the sample might be responsible for the difference in activity observed between sample MH and samples ML-1 and NC-1.

Samples ML-1 and NC-1 were both dissolved in ethanol and precipitated by the rapid addition of distilled water to obtain finely divided samples (ML-2 and NC-2). The dried samples were then evaluated against P388 leukemia in the same manner as the original samples. The new samples (ML-2 and NC-2) both gave results equal to or better than those obtained with sample MH (*see* table 1).

These data appear to show the importance of physical state of CCNU to its activity, a result dependent on the fact that the drug is administered as an aqueous suspension because of its low water solubility. Although the animal data presented here do not firmly establish the conclusion that physical form has a greater effect on activity than toxicity, the inferior results at 100 mg/kg in most cases, in terms of lifespan of dying animals or number of cures, indicate that this is the case.

Since CCNU (Lomustine) is normally administered orally to patients, one of the samples described above, ML-1, and its reprecipitated form were evaluated both orally and ip against the P388 leukemia in a second experiment. The results (table 2) were quite consistent with those obtained with ip administered drug in the first experiment.

MATERIALS AND METHODS

Carboxymethyl cellulose (0.4%).—The sodium salt of carboxymethyl cellulose (CMC) ether (Mathusen, Coleman and Bell) (4 g/liter) is mixed as thoroughly as possible with physiologic saline in a blender. To achieve the mixture, we autoclaved in a large vessel at 121°C for 45 minutes. The cooled solution is divided into small portions (50 ml/bottle) and autoclaved again at 121°C for 20 minutes.

Suspensions of CCNU.—A weighed amount of CCNU was mixed just prior to injection with 0.4% CMC using a Potter-Elveham tissue grinder driven with a hand drill mounted in a press.

Lymphocytic P388 leukemia.—The chemotherapeutic tests were carried out essentially as described (2). The data in table 1 were obtained in a single experiment and the data in table 2 in a second single experiment.

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Effect of Gentamicin and Irradiation on the Toxicity of High-Dose Methotrexate in Rats[1,2]

Brief Reports and Preliminary Communications

Gary B. Spector, Yeu-Ming Wang,* Chester A. Gleiser, Rafael C. Chan, and Jan van Eys[3]

With the increasing sophistication of oncology, the combination of chemotherapy, radiotherapy, and other modalities in the intensive treatment of neoplastic disorders has become routine in many cancer centers. Recently, methotrexate (MTX) at doses of ≥ 200 mg/kg has been used. A major side effect is renal toxicity. The combination of high-dose MTX and spinal irradiation in the management of leukemia, lymphoma, medulloblastoma, and, recently, osteosarcoma raises questions about changes in drug clearance. Furthermore, many patients receiving MTX subsequently develop signs or symptoms of infection.

Antibiotic therapy usually includes aminoglycosides. A few patients treated with MTX and irradiation or MTX with gentamicin have developed significant nephrotoxic effects, despite normal pretreatment serum creatinine and BUN levels. Experiments were designed to mimic the clinical situation to investigate the potential additive effects toward nephrotoxicity in these treatment combinations. This paper presents some preliminary findings on serum creatinine levels and MTX concentrations after such treatment regimens in rats.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200–250 g were used throughout the study. The animals were fed standard rat chow. Sodium bicarbonate (50 mEq/liter) was added to all drinking water to approximate the human protocol for high-dose MTX. Thirty-six rats were divided into six groups of six each: control, MTX alone, gentamicin alone, irradiation alone, gentamicin with MTX, and irradiation with MTX. MTX at a dose of 200 mg/kg (1.2 g/m²) was dissolved in 2 ml of 5% glucose in water and was infused through a tail vein over a 1-hr period. Citrovorum factor (CF) (100 mg/m²) was administered iv daily, again through a tail vein, beginning after MTX treatment and continuing for 7 days. The animals receiving gentamicin were treated according to the regimen developed by Cohen et al (1) for Sprague-Dawley rats, which required gentamicin at a dose of 100 mg/kg/day sc in two divided doses daily for 7 days. In a rat receiving MTX (200 mg/kg) and gentamicin (100 mg/kg/day), two drugs were given together on Day 1, and gentamicin and CF were continued over 7 days. Rats irradiated on the Cobalt-60 Theraton-80 machine received a dose of 500 rads to the spinal axis on 2 consecutive days. A field size of 10.5 × 1.0 cm was used at 80 cm source-to-skin distance, and was blocked with 5-cm-thick lead shields on the lateral border. The dose rate at d-max was 91.9 rads/min; the treatment time was 5.61 mins. The last group received irradiation in conjunction with a 1-hr infusion of MTX. The MTX was given 36 hrs after the completion of the irradiation. Again, CF was administered.

In three or four animals in each group serum creatinine levels were determined 7 days prior to the experiment, at 0 and 3 hrs after MTX, and daily thereafter. At 3, 24, 48, and 72 hrs, serum MTX concentrations were also quantitated by an enzymic inhibition assay (2). On Day 8 the surviving animals were killed for microscopic examination of the kidneys.

RESULTS AND DISCUSSION

The changes in serum creatinine levels for the various groups are shown in table 1. All six rats receiving 200 mg/kg of MTX and CF rescue survived the 7 days of observation. Serum creatinine concentration rose with a peak at 24 hrs, and creatinine levels returned to within the normal range

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TABLE 1.—Serum creatinine concentration in rats after high-dose MTX, gentamicin, irradiation, and combination therapies

Group	Serum creatinine concentration at—			
	0 hr	48 hrs	96 hrs	144 hrs
Control (4)*	1.31 ± 0.25†	1.32 ± 0.27	1.45 ± 0.28	1.45 ± 0.30
MTX (4)	1.51 ± 0.28	2.23 ± 0.60‡	1.70 ± 0.46	1.69 ± 0.30
Gentamicin (3)	1.23 ± 0.13	1.72 ± 0.05§	2.19 ± 0.96	2.07 ± 0.70
Irradiation (3)	1.27 ± 0.29	1.55 ± 0.30	1.33 ± 0.28	1.41 ± 0.40
MTX and gentamicin (4)	1.45 ± 0.34	2.20 ± 0.65‡	2.28 ± 0.72§	2.23 ± 0.90
MTX and irradiation (3)	1.20 ± 0.20	2.32 ± 0.26‡	1.61 ± 0.14	1.55 ± 0.19

*Nos. in parentheses = No. of observations.
†Mean ± 1 SD, in mg/dl.
‡Student's t test as compared to the control: $P < 0.025$.
§$P < 0.05$.

TABLE 2.—Serum MTX concentration in rats after high-dose MTX, MTX and gentamicin, and MTX with spinal irradiation

Treatment	Serum MTX concentration at—			
	3 hrs*	24 hrs†	48 hrs‡	72 hrs‡
MTX	4.18 ± 1.05	1.88 ± 0.47	2.57 ± 0.98	1.22 ± 0.33
MTX with gentamicin	7.12 ± 2.09§	3.43 ± 1.12§	3.19 ± 0.44	1.25 ± 0.41
MTX with irradiation	2.54 ± 0.55§	0.92 ± 0.34	6.63 ± 2.28§	3.84 ± 0.75¶

*Values in M × 10^{-4}, mean ± SD calculated from 3 observations.
†Values in M × 10^{-6}, mean ± SD calculated from 3 observations.
‡Values in M × 10^{-7}, mean ± SD calculated from 3 observations.
§$P < 0.005$.
¶Student's t test between MTX and MTX with gentamicin and between MTX and MTX with irradiation: $P < 0.05$.

by 96 hrs. The rats receiving gentamicin alone did not show a rise in serum creatinine until 48 hrs. This rise in serum creatinine then persisted throughout the 7 days of injections, but it was not statistically significant compared with the levels for the control animals.

One rat treated with both MTX and gentamicin died after 100 hrs of therapy. The serum creatinine in this group showed a significant increase over baseline throughout the experimental period. The animals receiving two doses of irradiation alone showed no increase in serum creatinine. However, at the same period a twofold increase in serum creatinine was seen in the animals receiving both irradiation and MTX 48 hrs from the start of MTX infusion. Table 2 shows serum MTX concentrations. It appears that gentamicin affected the α-phase and radiation modified the β-phase of plasma MTX elimination in these rats. This resulted in a decrease of the apparent volume of distribution of MTX in gentamicin-treated rats, and an increase of the apparent volume of distribution of MTX in rats treated with irradiation.

Necropsy was performed with special attention to the kidneys. Only three of the six rats receiving MTX alone demonstrated any renal lesions, and those showed only mild nephrosis. The changes were degenerative, involving the epithelium of the tubules in the upper portion of the medulla next to the cortex. There was desquamation of some epithelial cells and excessive granularity in others. Pyknotic nuclei were also observed. Amorphous material in the form of casts was observed in the lumina of some tubules. These histopathologic changes were diffuse and mild in severity.

At the time they were killed, all animals receiving only gentamicin were observed to be normal in appearance and activity. However, microscopic examination revealed that all animals had diffuse, severe necrosis of the tubular epithelium. Many tubular lumina appeared filled with proteinaceous casts. These changes were severe in the cortex, and were also observed in the medulla.

Of the six rats treated with both MTX and gentamicin, one died during the study and two appeared moribund at the time they were killed. As

in the animals treated with gentamicin, all had severe, diffuse nephrosis and they also demonstrated mild to moderate nephritis. The destructive changes were marked in both groups. However, additional inflammation was noted in the kidneys of MTX-gentamicin-treated rats. In animals treated with irradiation alone, the necropsies revealed normal tubular structure; however, the addition of MTX resulted in a microscopic picture similar to that observed with MTX alone in those animals that survived the treatment.

MTX is well-known for its nephrotoxicity (3). This drug and its metabolites are largely excreted by the kidney. Over 40% is excreted unchanged within 6 hrs of iv infusion and over 90% is excreted within 30 hours. Local renal damage decreases renal function, which results in diminished plasma drug excretion and prolongation of tissue exposure to MTX, and thus, further nephrotoxicity (4). This sequence is well-demonstrated in the results with high-dose MTX and gentamicin. The histopathologic findings support the chemical observations.

Reports in the literature describing significant nephrotoxicity from this antibiotic are sharply increasing. The damage credited to this aminoglycoside is necrosis of the proximal tubular cells (5). After one dose of gentamicin, the plasma MTX level is twice that in animals receiving MTX alone. A number of studies have shown that seemingly unrelated drugs may enhance the toxicity or decrease the potency of MTX (3). The findings described here suggest that gentamicin must be used with caution, if at all, in the presence of MTX. Although the use of gentamicin decreased the apparent volume of distribution of MTX in the rats, this fact may also suggest the localization of MTX in a few tissues. Under the conditions employed in this study, radiation alone does not impose a threat to the kidney, though a slow increment in the serum creatinine level was observed. Necropsy revealed that irradiation alone did not alter normal tubular structure in rats. However, it appears that radiation (on a field 10.5 × 1.0 cm) greatly alters normal rat tissue function, thus inducing the enhancement of MTX tissue distribution to a large degree. Two animals died by the 5th day after treatment with MTX and irradiation. An autopsy was performed in one of the two rats which showed a complete loss of intestinal epithelium. The animal had enteritis. There was severe congestion of the extraglomerular vessels. Some degenerative changes were seen in the lower nephron. No gross lesions were observed in the heart.

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Early Clinical Trial of a 1-Day Intermittent Schedule for Pentamethylmelamine[1,2]

Brief Reports and Preliminary Communications

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SUMMARY

An early clinical trial of pentamethylmelamine (PMM), the monodemethylated derivative of hexamethylmelamine, was conducted in 22 adults with solid tumors. PMM was administered as a 2-hour iv infusion every 4 weeks at doses ranging from 40 to 2400 mg/m². A combination of gastrointestinal and neurologic (CNS) toxicity was dose-limiting. Nausea and vomiting began at a dose of 265 mg/m² and became progressively worse until it became life-threatening at doses of 1800–2400 mg/m². CNS toxic effects consisting of agitation, confusion, drowsiness, and loss of consciousness were first noted at a dose of 1200 mg/m² and were seen in varying degrees at all higher dose levels. No other toxic effects were noteworthy except two instances of thrombocytopenia at low doses. No antitumor activity was observed. We do not recommend the further use of this schedule of administration for PMM.

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Hexamethylmelamine (HXM) was chosen for clinical trials based on its antitumor activity in a number of rodent tumors (1). Clinical trials of HXM demonstrated that several tumors, including small cell lung cancer, ovarian cancer, breast cancer, and lymphoma, responded favorably to treatment (2). HXM given orally produces significant, frequently treatment-limiting nausea and vomiting. Because of limited solubility in water and in nontoxic solvents, a parenteral preparation of HXM has not been used in human trials.

In studying the metabolism of HXM, Worzalla et al (3) identified a series of demethylated products. *N*-Demethylation increases water solubility and several demethylated products were tested for activity against the P246 tumor, a human bronchial carcinoma grown in immune-deprived mice. Of the metabolites examined, pentamethylmelamine (PMM) showed therapeutic effectiveness equivalent to HXM. PMM is approximately 24 times more soluble in water than HXM (4). Because of its activity against the human bronchial tumor, a parenteral preparation of PMM was developed for clinical use.

HXM and PMM have limited activity against a wide variety of the more standard animal tumor models.[4] PMM was also inactive against the human lung xenograft, LX-1, but was active against the MX-1 human breast cancer xenograft and the $CD8F_1$ mammary tumor. The mechanism of antitumor activity is unknown but it does not appear to be either alkylation (3) or folic acid antagonism.[5] In animal tumor systems, daily or intermittent 1-day treatment regimens were more effective than single or multiple doses on Day 1 only.

The preclinical toxicologic evaluation of PMM was conducted in mice, dogs, monkeys, guinea pigs, and rabbits. The following toxicity data are from dogs and monkeys. In both these species, the major clinical manifestations of drug toxicity occurred primarily during or immediately following drug administration and consisted of diarrhea, emesis, salivation, tetanic seizures, hyperactivity or hyperexcitability, sedation, depression, adipsia, anorexia, weight loss, hypothermia, weakness, and dyspnea.[4] The symptoms relating to the CNS stimulation were dependent on the rate of drug administration. Blood chemistries and clinical hematology were only transiently affected at sublethal doses and did not correlate well with the histologic findings.

Our early clinical trial of PMM in man was done using a 1-day intermittent dose schedule. The start-

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[4]Clinical brochure: pentamethylmelamine. Prepared by the NCI, Bethesda, MD, Sept 1979.

[5]Progress Report, No. 10. Southern Research Institute, Birmingham, AL, Nov 1977.

ing dose chosen was 80 mg/m² and represented approximately one third the toxic dose low in dogs on the 1-day treatment schedule. Because of the CNS symptoms noted in the preclinical studies following rapid iv administration, PMM was administered as a 2-hour iv infusion.

MATERIALS AND METHODS

Twenty-two adult patients with a variety of solid tumors were entered in the study after giving written informed consent. All patients had histologic or cytologic confirmation of advanced cancer without any reasonable hope of cure or significant palliation using conventional therapies. Contra-indication to entry in the study included leukopenia (wbc count < 4100/mm³), thrombocytopenia (platelet count < 130,000/mm³), hemoglobin < 10 g/100 ml, any elevation of direct bilirubin, serum creatinine ≥ 1.5 mg/dl, an Eastern Cooperative Oncology Group (ECOG) performance score (PS) of 4, major surgery within 30 days, significant (ie, > 2500 rads to entire pelvis) or recent (< 30 days) radiation therapy, significant infection, frequent vomiting, and severe anorexia.

Pretreatment tests included a medical history and physical examination, standard hematology and blood chemistry profiles, serum amylase, prothrombin time, urinalysis, chest roentgenograms, and ECG. Electroencephalograms and electromyograms were not done routinely, but were obtained if certain toxic effects became apparent. Blood and urine specimens were drawn periodically before, during, and following the treatment for pharmacologic studies and those data have been reported elsewhere (5,6).

PMM is a white powder supplied in 500-mg vials, with mannitol (300 mg) and sodium hydroxide to adjust pH. The solution was prepared by reconstituting the powder with 9.5 ml of sterile water for a USP injection. The solution was then added to 500 ml of 5% dextrose in water and infused over 2 hours. Subsequent treatments were scheduled at 4-week intervals. Pulse, temperature, and blood pressure were determined prior to treatment, and then standing and recumbent pulse and blood pressure were determined at 15 minutes and every 30 minutes for 2 hours following drug administration. Patients were routinely questioned about acute toxicity 24 hours after their treatment.

The dose levels (mg/m²) tested were 40 (dose reduction for thrombocytopenia at 80), 80, 160, 265, 400, 600, 800 (dose reduction for severe nausea and vomiting), 1200, 1800, and 2400.

The median age of the 22 patients treated was 58 years (range, 22–77). There were 15 males and seven females. Ten patients had an ECOG PS of 0–1 and 12 had a PS of 2–3. Nineteen of the 22 patients had had prior chemotherapy, 14 had had prior radiation therapy, 13 had received both chemotherapy and radiation therapy, and only two had received neither. Tumor types of the 22 patients treated included lung cancer (one adenocarcinoma, two large cell, and two small cell), gastrointestinal tumors (four of colon and one each of esophagus, duodenum, appendix, and rectum), squamous cell cancers of the head and neck (two), and other various tumors including one each of breast, squamous cell skin, melanoma, pleural mesothelioma, carcinoid, carcinosarcoma, and adenocarcinoma of unknown primary. Of the 22 patients, all are evaluable for acute toxicity, and 15 were evaluable for at least 4 weeks following treatment.

Toxic Effects

Two patients received initial treatment at the 1200- and 2400-mg/m² dose levels, and three were initially treated at each of the other scheduled dose escalations. In all, 32 treatments were given: 22 as first courses, six as second courses, three as third courses, and one as a fourth course.

Hematologic toxicity was very infrequent. No leukopenia or definitely drug-induced anemia was encountered. Two apparently drug-induced episodes of thrombocytopenia occurred, one at 80 mg/m² as a second course and one at 265 mg/m² as a first course (nadirs of 48,000 and 70,000 cells/mm³ on Days 16 and 27, respectively). No definite renal or hepatic toxicity was noted. No alopecia was observed.

Principal and dose-limiting toxic effects were confined to the gastrointestinal system and the CNS. No toxicity was noted until the 265-mg/m² dose level when mild to moderate vomiting occurred. Severe vomiting began at a dose of 600 mg/m² and progressed to a life-threatening degree at doses of 1800 and 2400 mg/m² (prolonged vomiting leading to dehydration and hypotension requiring hospitalization) in one patient at each level. CNS symptoms were first noted at the 1800-mg/m² dose level. At that dose, one patient experienced moderate agitation for 24 hours and one had moderate confusion for 4 hours. One of the two patients scheduled to receive the 2400-mg/m² dose became unconscious and was not arousable 1 hour after receiving 50% (1200 mg/m²) of the infusion. Moderate elevation of blood pressure (50 mm Hg rise in both diastolic and systolic pressures) also occurred. The infusion was stopped and the patient regained consciousness over 30 minutes but remained confused for almost

12 hours more. The other patient at the 2400-mg/m² dose level experienced mild drowsiness for 12 hours in addition to severe nausea. Electroencephalograms done on both patients scheduled at the 2400-mg/m² dose level 24 hours after treatment were unchanged from their pretreatment baseline. No cumulative neurologic toxic effects were noted in the seven patients who received more than one course of PMM at levels producing no immediate neurologic toxicity.

In summary, gastrointestinal toxic effects (nausea and vomiting) began at 265 mg/m², were moderate to severe at 600–1200 mg/m², and were frequently life-threatening at 1800 and 2400 mg/m². CNS symptoms of agitation, drowsiness, confusion, and loss of consciousness were first seen at a dose of 1200 mg/m² (one half of the planned 2400-mg/m² infusion), in two of three patients at 1800 mg/m², and in the only patient receiving 2400 mg/m². At a dose of 2400 mg/m², we felt the combined nausea and vomiting and CNS symptoms were dose-limiting and stopped further patient entry in the study. There were no drug-related deaths.

Therapeutic Effects

No definite tumor regressions were noted. One patient with adenocarcinoma of the colon had an "improvement" in liver size and liver function tests for 2 months. Fifteen patients had disease progression or died after the first treatment, four after the second, two after the third, and one after the fourth.

DISCUSSION

A 2-hour infusion of PMM at doses of 40–2400 mg/m² in man produced gastrointestinal and CNS side effects predicted by the preclinical models. At doses of ⩾ 1800 mg/m², severe, prolonged nausea and vomiting became clinically intolerable. CNS symptoms began at a dose of 1200 mg/m² and were seen in varying degrees in the majority of patients at higher dosages.

PMM produces nausea and vomiting comparable to that seen with HXM. In addition, both drugs produce CNS toxicity, that of PMM being more acute. The 2400-mg/m² dose of PMM represents approximately 25% of one of the more common schedules (6) for HXM (12 mg/kg or 480 mg/m² daily × 21 days = 10,080 mg/m²).

Because significant toxicity was noted on the 1-day intermittent schedule for PMM at doses up to 2400 mg/m² given as a 2-hour iv infusion, we suggest this schedule not be employed in any phase II evaluation of PMM.

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Chemotherapy for Small Cell Bronchogenic Carcinoma: CCNU and Doxorubicin Compared to CCNU, Doxorubicin, Vincristine, and Procarbazine[1,2,3]

Brief Reports and Preliminary Communications

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SUMMARY

Forty consecutive patients with small cell bronchogenic carcinoma were treated. The first 18 patients were treated with CCNU and doxorubicin (Adriamycin) (CA). The next 22 patients were treated with CCNU, doxorubicin, procarbazine, and vincristine (CAPO). Patient characteristics were similar. The partial plus complete response rate was 55% (ten of 18 patients) in the CA group compared to 41% (nine of 22 patients) in the CAPO group. The median survival from treatment was 28 weeks in the CA group compared to 33 weeks in the CAPO group. There were no drug-related deaths among the patients receiving CA compared to two definite and three probable drug-related deaths among the patients receiving CAPO. The addition of procarbazine and vincristine to CA for the treatment of small cell bronchogenic carcinoma resulted in increased toxicity and no survival benefit.

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Small cell bronchogenic carcinoma is sensitive to multiple chemotherapeutic agents (1,2). At the University of Minnesota the use of CCNU plus doxorubicin (Adriamycin) (CA) in the treatment of small cell carcinoma of the lung resulted in an overall response rate of 63% (3). In an attempt to increase the response rate and to prolong survival, procarbazine and vincristine were added to the CA combination.

The results of treatment of small cell carcinoma of the lung with CCNU, doxorubicin, procarbazine, and vincristine (CAPO) were compared to the previous study using CA.

MATERIALS AND METHODS

Forty consecutive patients with histologically proven small cell bronchogenic carcinoma were entered in this study between July 1974 and November 1977. The first 18 patients were treated between July 1974 and October 1976 with CA. Preliminary data on 15 patients have been reported (3). The next 22 patients were treated between October 1976 and November 1977 with CAPO. There were no patient exclusions for age, performance status, or expected survival. Patients with congestive heart failure were excluded. No patients with prior chemotherapy were entered in the CAPO study; three patients in the CA study had received prior chemotherapy. All pathologic material was reviewed in 1978 and confirmed as being small cell carcinoma.

The pathologic review included all available specimens. The tumors were classified according to the Working Party for Therapy of Lung Cancer (4). Three types of small cell carcinoma were recognized: the classic oat cell or lymphocyte-like type;

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the intermediate type (including variants designated as fusiform or polygonal); and a mixed type which included small cell carcinoma associated with other histologic types.

Patients were evaluated for the presence of extrathoracic tumor by liver and bone scans, bone marrow biopsy, and lumbar puncture. Computerized axial tomography of the brain was performed on all patients who received CAPO. After these procedures, 28% of the CA group and 32% of the CAPO group were found to have limited disease.

The CA group received CCNU (100 mg/m²) on Day 1 and doxorubicin (60 mg/m²) on Day 1 with the cycle repeated every 6 weeks. The CAPO group received CCNU (100 mg/m²), doxorubicin (50 mg/m²), and vincristine (1 mg/m²) on Day 1. Procarbazine (100 mg/m²) was given orally for the first 14 days and the cycle was repeated in 6 weeks. Drug doses were modified to maintain the wbc count between 2000 and 4000/μl and the platelet count at > 75,000/μl.

After three courses of either regimen, all disease parameters were re-evaluated. If no evidence of disease outside a single thoracic radiotherapy port was found, patients received 4000 rads over 4 weeks to the primary lung lesion and adjacent involved nodal areas. The fourth course of chemotherapy (less doxorubicin) was given concurrently with the irradiation therapy. No prophylactic CNS radiotherapy was given.

Tumor response to chemotherapy was evaluated by standard response definitions (3). Any response less than a partial response was considered no response.

RESULTS

The patient characteristics of the two groups were comparable (table 1). Eighteen patients were treated with CA. All patients have died of their disease, and all patients were evaluable for response and survival. The overall objective response rate before radiotherapy was 55% (ten of 18 patients). The median duration of response to CA was 149 days (4.9 months). Five patients (28%),three with limited disease, had a complete response. Their median survival from the start of treatment was 12 months. Five patients, all with extensive disease, had a partial response, and their median survival was 6.5 months. Eight patients had no response and a median survival of 5.2 months. The overall median survival from treatment for all patients treated with CA was 6.5 months.

Twenty-two patients were treated with CAPO. Twenty-one of the 22 patients have died. Two patients died at Days 10 and 15 of therapy and are not evaluable for response but are evaluable for survival. The overall objective response rate before radiotherapy was 41% (nine of 22 patients). The median duration of response to the CAPO combination was 106 days (3.5 months). Two patients (10%), both with limited disease, had a complete response. One patient died while in remission at 8 months. The other patient is currently living after 30 months without receiving any therapy. Seven patients had a partial response and a median survival from start of treatment of 12.3 months. Thirteen patients had no response and a median survival of 5 months. The overall median survival of all patients treated with CAPO was 7.7 months.

In the combined groups totaling 40 patients, the overall response rate was 50%, with a median survival from treatment of 7.2 months. Limited disease did not predict for response or survival.

The toxic effects of the two regimens were different. All patients receiving CA experienced nausea, vomiting, and alopecia. There were no drug-related deaths. Hematologic toxic effects were mild

TABLE 1.—Patient characteristics

Characteristic	No. of patients in—	
	CA group	CAPO group
Total	18	22
Mean age (yrs) 62.6 (CA group) 61.7 (CAPO group)		
Men	12 (66%)	17 (77%)
Limited disease	5 (28%)	7 (32%)
Karnofsky performance status		
8–10	11 (61%)	15 (68%)
5–7	7 (39%)	7 (32%)
1–4	0	0

TABLE 2.—Comparison of 2-drug to 4-drug chemotherapy

Parameters	Regimen	
	CA	CAPO
No. of patients	18	22
Median survival from diagnosis (mos)	8	8
Median survival from treatment (mos)	6.5	7.7
% response rate (complete + partial)	55	41
% complete responses	27	10
Median survival from diagnosis of patients with complete or partial response (mos)	12.0	13.7

with some cumulative leukopenia and thrombocytopenia. Nadir wbc counts of < 1000/μl were not documented.

Patients receiving the four-drug CAPO combination also experienced nausea, vomiting, and alopecia. Procarbazine was discontinued in eight patients after a median of four courses because of nausea, vomiting, and/or leukopenia. Hematologic toxicity was severe. Two patients died of leukopenia and sepsis within 15 days of initiation of therapy, and their deaths are considered to be drug-related. Three additional patients died at Days 105, 180, and 250 of therapy. The exact causes of death were undetermined, but they were possibly drug-related. Nadir wbc counts of < 1000/μl occurred in three other patients. All patients experienced progressive anemia, leukopenia, and thrombocytopenia, resulting in downward adjustments in drug dose.

The various histologic subtypes did not influence response to therapy or survival. Twenty-nine patients with the lymphocyte-like subtype had a 45% response rate and a median survival from diagnosis of 8.1 months. Eleven patients with the intermediate subtype had a 55% response rate and a median survival from diagnosis of 7.4 months. This confirms the prior data of Matthews (5).

DISCUSSION

Reports on several series of patients with small cell bronchogenic carcinoma treated by various regimens have been published recently. Hansen et al (6) reported a 78% response rate and a 33-week median survival in 53 patients treated with CCNU, cyclophosphamide, methotrexate, and vincristine every 4 weeks. Greco et al (7), using cyclophosphamide, doxorubicin, vincristine, VP-16-213, and hexamethylmelamine every 3 weeks reported complete remissions in 29 of 32 patients (91%) with limited disease. Median survival is currently 70 weeks. Cohen et al (2) reported complete remissions in 14 of 19 patients (74%) with limited disease and a median survival of 61 weeks. Chemotherapy was given every 3 weeks, except for CCNU which was given every 6 weeks.

Compared to these series, the combined CA and CAPO groups had a lower overall response rate and median survival. Therapy given every 6 weeks may not be adequate treatment for a tumor with a high growth fraction. These drug regimens (CA and CAPO) therefore appear somewhat less effective than other three- and four-drug regimens.

In this nonrandomized retrospective study, the four-drug CAPO regimen was not superior to the two-drug CA regimen (table 2). In addition, there was an increased toxicity with the CAPO regimen compared to the CA regimen.

Vincristine is an active agent in small cell carcinoma. Dombernowsky et al (8) used vincristine as a single agent in 19 patients and reported a 42% response rate. Further demonstration of the activity of vincristine was provided by Hansen et al (6). In a randomized study, vincristine increased survival when added to the combination of CCNU, cyclophosphamide, and methotrexate. In the current CAPO regimen, the administration of vincristine every 6 weeks may have been too infrequent to make an impact on the response rate or survival.

The role of procarbazine in the combination therapy for small cell carcinoma is less well-defined. The 47% response rate of procarbazine as a single agent is based on studies in nine patients (9). Multiple regimens containing procarbazine have been reported but each regimen contains other effective agents, and the additional benefit of the procarbazine is difficult to assess. In this study the addition of procarbazine to CA did not improve the response rate and resulted in increased toxicity.

In conclusion, the combinations of CA or CAPO

appear to be less effective when compared to other three- and four-drug regimens. The addition of vincristine and procarbazine to the two-drug combination of CA resulted in increased toxicity and no survival benefit. Since neither this study nor prior studies adequately substantiate the effectiveness of procarbazine in the treatment of small cell bronchogenic carcinoma, its role should be re-evaluated.

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Phase II Trial of Vindesine in the Treatment of Lymphomas, Breast Cancer, and Other Solid Tumors[1,2]

Brief Reports and Preliminary Communications

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SUMMARY

A phase II study of vindesine in 41 evaluable patients demonstrated the drug to be active in heavily pretreated patients with breast cancer, non-Hodgkin's lymphoma, and other tumors. There were two partial responses in 11 patients with breast cancer (18%) and five partial responses and one complete response in 11 patients with non-Hodgkin's lymphoma (40%). Other responses were seen in small cell carcinoma of the lung, ovarian carcinoma, and Hodgkin's disease. Prior vinca exposure did not adversely affect the response rate. Neutropenia was dose-limiting. Neurotoxic effects occurred in 10% of the patients. A high incidence of local tissue reactions at the injection site (27%) could be reduced by a careful administration technique. Vindesine should be studied further in combination with other agents.

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Vindesine is a new vinca alkaloid derived from vinblastine. It has a wide spectrum of antitumor activity in animal test systems (1). Vindesine is currently undergoing extensive phase II testing and has been shown to be active in a number of human tumors (1–7). The present phase II trial further examines the activity of vindesine in a variety of adult tumors.

PATIENTS AND METHODS

Between May 1978 and September 1979, 45 patients with advanced carcinoma (32 patients) and lymphoma (13 patients) were treated in a phase II trial of vindesine at the University of Arizona and the Tucson Veterans Administration hospitals. Of the 45 eligible patients, 41 were evaluable for response and four were not evaluable (one patient refused therapy, one did not have measurable lesions, and two received inadequate therapy). All 41 patients had measurable disease and had previously failed other chemotherapy regimens. No patient received concomitant radiotherapy.

The initial dose of vindesine[4] was 3 mg/m^2 administered by slow iv injection every week until disease progression was documented. The initial vindesine dose was reduced by 50% in cases of impaired bone marrow and in patients > 65 years old. Appropriate reductions in dose were made if significant myelosuppression occurred.

Initially, abnormal studies were repeated periodically to evaluate response. A complete response (CR) was defined as the disappearance of all measurable disease. A partial response (PR) was defined as a > 50% reduction in the product of two perpendicular tumor diameters of all evaluable lesions without evidence of progression in other sites. Progressive disease (PD) was defined as any increase in tumor dimensions. Freedom from relapse for responders was calculated as the time from initiation of treatment to disease progression.

RESULTS

Forty-one patients were evaluable for response and toxicity. Of 15 patients with advanced breast carcinoma (table 1), objective responses (PRs) were observed in two of 11 (18%) evaluable patients, and minimal response (MR) occurred in two others.

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[4]Vindesine was supplied by the Eli Lilly Co, Indianapolis, IN.

TABLE 1.—Patient characteristics and results of vindesine treatment in patients with breast cancer and lymphoma

Characteristic	Patients with breast cancer	Patients with non-Hodgkin's lymphoma
No. of evaluable patients	11	11
Previous chemotherapy		
Mean No. of drugs (range)	6.3 (3–10)	5.8 (1–12)
Mean No. of drug combinations (range)	3.0 (1–5)	2.3 (1–6)
No. with prior vincristine exposure	8	9
No. with prior vinblastine exposure	0	4
Response		
CR	0	1
PR	2	5
MR	2	2
PD	10	3
Median freedom from relapse in wks (range)	13 (4–18)	6 (4–36)

Median freedom from relapse for the four patients with PR or MR was 13 weeks (range, 4–18 weeks). Of 11 patients with stage IV non-Hodgkin's lymphoma refractory to standard chemotherapy regimens (table 1), objective responses occurred in six patients (55%) (one CR and five PRs); there were two MRs. These responses and improvements were seen in all of five patients with nodular histologic types and in two of six with diffuse histologic types. Median freedom from relapse was 6 weeks (range 4–36 weeks). Responses were also noted in one of two patients with Hodgkin's disease (PR), in one of three with ovarian carcinoma (MR), and in one with small cell carcinoma of the lung (PR). A number of tumor histologic types did not show an objective response to vindesine: multiple myeloma (four patients), malignant melanoma (three), adenocarcinoma of the lung (two), endometrial carcinoma (one), liposarcoma (one), and malignant fibrous histiocytoma (one).

Previous vinca exposure did not adversely affect the response rate. Eleven of 27 patients (41%) previously exposed to vincristine and two of six (33%) previously exposed to vinblastine responded to vindesine. Eight of 22 patients (36%) having PD while receiving a vincristine-containing drug combination had an objective response to vindesine. Twelve patients had never received a vinca alkaloid prior to vindesine, and four (33%) of these patients had objective responses.

Myelosuppression was the most common toxic effect with the dose schedule used. Leukopenia (< 3000 cells/mm^3) occurred in 28 of 41 patients (68%), and 24 of those patients required either a dose reduction or an attenuation of the schedule to every other week. Thrombocytopenia (< 100,000 platelets/mm^3) was an uncommon side effect (10%). Anemia (decrease of 1 g of hemoglobin/dl/week) was relatively common, occurring in 15 patients (37%).

A common side effect of vindesine in this series was local tissue injury following injection. Cellulitis, phlebitis, and inapparent infiltrations with subsequent tissue necrosis were seen in 11 patients (27%). Inapparent infiltrations appeared to occur more commonly with vindesine than with the other vinca alkaloids (8). For example, during the first 3 months of the study five of 34 vindesine injections resulted in local tissue injury from inapparent infiltrations. During the same time period, in the same clinic, with the same personnel, no infiltrations were seen in 231 injections of vincristine and in 46 injections of vinblastine. The higher incidence of local infiltrations with vindesine was significant ($P = 0.001$). Because of this incidence of inapparent infiltrations, we changed our administration procedure to include an infusion of 50 ml of 5% dextrose in water prior to a slow injection of vindesine, followed by another 50 ml of 5% dextrose in water. This change in administration technique resulted in a decreased number of inapparent infiltrations (two infiltrations occurred during the next 115 administrations, $P = 0.01$).

Other side effects less frequently encountered included paresthesias (four patients), myalgias (four), motor weakness (three), hair loss (two), stomatitis (one), fever (one), chills (one), nausea and vomiting (one), and fatigue (one). The neurologic side effects resolved with discontinuation of vindesine treatment or a decrease in the vindesine dose schedule.

CONCLUSIONS

Vindesine produced objective results in patients heavily pretreated for breast cancer, lymphoma,

and small cell carcinoma of the lung. The response duration was brief. Prior vinca exposure did not adversely affect the response rate. Neutropenia was the dose-limiting toxic effect in most patients. The number of local tissue reactions to vindesine injections suggests that extra care should be exercised during its administration. The frequency of these tissue reactions can be reduced with a proper administration technique (8).

Data from this study and other recent reports (1–7) indicate that vindesine is a useful anticancer drug and further investigation of vindesine in combination with other agents is justified.

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Chemotherapy for Advanced Cervical Cancer With Bleomycin, Vincristine, Mitomycin C, and *cis*-Diamminedichloroplatinum(II) (BOMP)[1,2]

Brief Reports and Preliminary Communications

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The demonstration that *cis*-diamminedichloroplatinum(II) (CDDP) is the single most active drug in the chemotherapy for advanced squamous cell cancer of the uterine cervix (1,2) opened the way to the design of regimens combining this agent with others of known activity.

The initial experience with a combination of CDDP, methotrexate (MTX), and bleomycin (MBD) demonstrated a high order of activity (eight objective responses among nine treated patients) (3). However, toxicity was unacceptable and thought to be related to impaired excretion of MTX in patients with pelvic tumor obstructing the ureters (3). The same MBD regimen has been both tolerable and effective for treatment of advanced squamous cell cancers of the head and neck (4) and esophagus (5).

The present study was a pilot project to develop a tolerable combination of CDDP (P) with three drugs shown to be active, singly and in combination, in cervical cancer: mitomycin C (M), vincristine (O), and bleomycin (B) (MOB). The response rate to the combination of the three drugs alone is reported to be 45%, with some apparent advantage, in a nonrandomized comparison, for a semi-weekly schedule of bleomycin and vincristine or a bleomycin infusion (6). In the present trial, we chose a schedule of the MOB drugs involving weekly administration of vincristine and bleomycin because we expected it to be both more tolerable and a great deal more convenient for outpatient administration than schedules involving twice weekly administration or continuous infusions. We call our regimen "BOMP."

MATERIALS AND METHODS

Sixteen patients with advanced cancer of the uterine cervix and measurable areas of tumor were entered in the study. Three of the 16 patients were excluded from further analysis: one left the country 3 weeks after starting chemotherapy, one had marked pain relief but refused both further therapy and repeated computerized tomography to evaluate response, and one had prompt marked regression of a pelvic mass but refused to return for medical care while febrile 2 weeks after her first dose. She died at home perhaps due to an infection complicating treatment-induced myelosuppression (her platelet count had fallen to 65,000/μl 10 days after treatment, although her wbc count was still 5100/μl at that time).

The median age of the 13 remaining assessable patients was 50 years (range, 39–74). Seven patients were black and six were white. Four patients were asymptomatic, five were ambulatory with symptoms, two were bedridden less than one half of the day, and two were bedridden more than one half of the day. Two patients had failed prior chemotherapy with ICRF-159; the remaining 11 patients had received no prior chemotherapy. Three patients had major prior surgery, and 11 had progressive tumor after radical irradiation. Two patients (stage IIIB and IVA) were given the BOMP regimen electively prior to planned radiation therapy in the hope of shrinking the tumor and making subsequent radiotherapy more effective.

Tumor was present in the pelvis in nine patients, in the lung in five, in the abdomen or retroperitoneal nodes in four, in the peripheral nodes in three, and in the bone and liver in one patient each. No patient had bone involvement alone. The histologic type was squamous cell cancer in all but a single

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patient who had adenosquamous carcinoma. The median interval from diagnosis of cervical cancer to BOMP chemotherapy was 11 months, with a range of 0–25 months.

The BOMP regimen was given on an outpatient basis in 6-week cycles. Bleomycin was given im at a dose of 10 units on Day 1 and weekly. Vincristine was given iv at a dose of 1.0 mg/m^2 on Days 1, 8, 22, and 29; mitomycin C was given iv at a dose of 10 mg/m^2 on Day 1; and CDDP was given iv at a dose of 50 mg/m^2 on Days 1 and 22. CDDP was given 30 minutes after starting a 2-hour infusion of 2 liters of 5% dextrose in 0.45% sodium chloride containing 10 mEq KCl/liter. Furosemide (40 mg) was given iv at the start of the infusion, and mannitol (12.5 g) was given iv just prior to the CDDP. This regimen of CDDP administration has proved safe and effective for outpatient use (7).

Doses of mitomycin C and CDDP were reduced by 50% for a wbc count of 3000–4000/μl or a platelet count of 75,000–100,000/μl. These drugs were withheld if blood cell counts fell below these levels. Vincristine was discontinued for peripheral neuropathy if functional impairment was present.

Partial remission was defined as a 50% decrease in the sum of the products of perpendicular diameters of measurable lesions. Abdominal and pelvic tumors were followed by palpation, ultrasound, and computerized tomography. Partial remission was considered terminated when new lesions appeared, when there was a 25% increase in the estimated cross-sectional area of measurable lesions, when there was a major deterioration in performance status (more than one level on the Eastern Cooperative Oncology Group scale), or when there was recurrence of disease-related symptoms such as pain. Remission duration and survival were calculated from the start of treatment.

RESULTS

Ten of 13 patients achieved remissions that were at least partial, and in three patients all clinical evidence of disease disappeared. In all patients these objective responses were accompanied by major symptomatic improvement. This was usually apparent within the first week of treatment. The median duration of response is 4 months (range, 2.5–6+ months). Two patients were electively referred for pelvic radiotherapy during a BOMP-induced remission; one remains well and free of palpable disease 10 months from diagnosis. The other patient had disease progression during radiotherapy and died 4 months after entry in the study. The cause of death was interstitial pneumonitis possibly related to ethiodol administration for a lymphangiogram. The histologic pattern of pneumonitis at autopsy excluded bleomycin lung toxicity in this patient. The pneumonitis had developed 2 days after ethiodol injection, and after a cumulative bleomycin dose of 110 units over 12 weeks.

The median projected survival for all patients from time of entry in the study is 8 months. The nonresponders survived 3, 4, and 8 months respectively; the median projected survival of responding patients is 10 months (based on six deaths among ten responders). All three nonresponding patients had disease in the pelvis, accompanied by paraaortic nodes in one and bone involvement in another.

Toxicity was generally acceptable. No patient developed a wbc count < 2000/μl, and only three patients ever had a wbc count between 2000 and 3000/μl. A single patient had a platelet count < 25,000/μl, and this was complicated by bleeding from radiation proctitis. Three additional patients had platelet counts between 25,000 and 50,000/μl unaccompanied by bleeding. Five patients required transfusions, and an additional five had hemoglobin levels that fell to < 9.5 g/dl. Peripheral neuropathy characteristic of vincristine toxicity developed in eight patients; it was mild in three and moderately severe in five. No patient developed stomatitis, clinically evident hearing loss, or azotemia. Hypomagnesemia was not routinely searched for and was not documented in any patient. Nausea and vomiting of moderate severity were universal.

DISCUSSION

The regimen described here (BOMP) has a high order of activity in advanced cervical cancer: a 78% objective response rate (63%, if all nonevaluable patients are considered nonresponders). Toxicity was acceptable, and the regimen is suitable for outpatient administration. Cervical cancer should now be considered a tumor in which chemotherapy is of established value. This value is severely limited, however, by the short duration of response (median, 4 months).

The requirement for all components of the BOMP regimen is now under examination in a Southwest Oncology Group trial which compares the single most active agent, CDDP, to combination mitomycin C plus CDDP and to all four drugs combined. In this trial, bleomycin is given as an infusion of 120 mg over 4 days every 6 weeks and vincristine at a total dose of only 1 mg/m^2 every 6 weeks (L. Baker, personal communication).

The results of the present trial, though based on a small patient sample, appear substantially superior to those achieved with high-dose MTX plus

vincristine (8) (a 17% response rate), doxorubicin as a single agent (a 16% response rate) (9), doxorubicin plus MTX (a 27% response rate) (10), and doxorubicin plus methyl-CCNU (a 45% response rate) (11). Results of combining bleomycin with low-dose, biweekly, oral MTX were more encouraging, with a 60% response rate and a median duration of response of 7.5 months (12). Higher parenteral doses of MTX, however, appear quite dangerous in patients with this disease, although activity in combination with bleomycin and CDDP is very high (5).

The major use of the BOMP regimen, in view of the short duration of response in patients with recurrent disease after radiation therapy, may prove to be as initial treatment for locally advanced cervical cancer prior to planned radiation therapy. This program was well-tolerated in two patients in the present series.

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Phase II Trial of 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (AMSA) in Patients With Metastatic Renal Cell Carcinoma[1]

Brief Reports and Preliminary Communications

David A. Van Echo,* Susan Markus, Joseph Aisner, and Peter H. Wiernik[2]

Metastatic renal cell carcinoma is unresponsive to most chemotherapeutic agents employed to date including alkylating agents (1–4), antimetabolites (2), antibiotics (3), vinca alkaloids, and nitrosoureas (5). Progesterone derivatives or androgens give a low number of partial remissions and approximately a 20% subjective response rate (1,6–8). The current phase II study was undertaken to evaluate the therapeutic efficacy of a new investigational agent, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (AMSA) in this disease.

MATERIALS AND METHODS

Patients with histologically documented metastatic renal cell carcinoma who were admitted to the Baltimore Cancer Research Program were eligible for this study. Patients were expected to have bidimensionally measurable disease by routine clinical or laboratory testing, at least a 2-month life expectancy, adequate organ function (serum creatinine $\leq$ 1.5 mg/dl, bilirubin $\leq$ 1.5 mg/dl), and hematologic counts (wbc count $\geq$ 4000/μl, platelet count $\geq$ 100,000/μl). All patients entered gave informed consent.

Standard criteria for response were employed: complete remission (CR)—disappearance of all demonstrable lesions and normalization of all abnormal laboratory values for at least 1 month; partial response (PR)— > 50% decrease in the sum of the products of diameters of all measurable lesions for at least 1 month; stabilization—< 50% reduction, and tumor size remaining unchanged during at least 2 months of therapy; progression— $\geq$ 25% increase in the sum of the products of the diameters of any measurable lesions or the appearance of any new lesions outside of the CNS. Any patient who developed new CNS metastases was given whole-brain irradiation, 3000 rads in 10 fractions over 2 weeks. Such patients were continued in the protocol until there was evidence of progression outside the CNS.

AMSA was administered iv at a dose of 120 mg/m² as a 60-minute infusion diluted to a volume of 250 ml in 5% dextrose and water. Therapy was repeated every 21 days for a minimum of two courses and was continued if the patient had improvement or stable disease until there was evidence of disease progression. Patients were seen at least weekly to assess any problems that may have arisen secondary to the patients' disease or therapy. Blood cell counts were performed two to three times per week and serum chemistries at least weekly. Dosage modifications were *not* made according to nadir cell counts but were based on the occurrence of hemorrhagic or infectious complications and on the basis of the blood cell counts on Day 21 of each course according to the following criteria. If at the end of 21 days no hematologic toxicity had occurred and the wbc count was $\geq$ 4000/μl and the platelet count was > 150,000/μl, the chemotherapy dose was increased 30 mg/m² for the next course; if by Day 21 the wbc count was 2000–2900/μl and/or the platelet count was < 100,000/μl, subsequent therapy was delayed for 1 week. AMSA was then given at 50% of the level of the prior course; if the bone marrow biopsy was positive for tumor prior to therapy, no dose modifications were made, regardless of hematologic toxicity, until the biopsy became negative.

RESULTS

Sixteen patients with histologically proven, metastatic clear cell renal carcinoma were treated with AMSA. All patients had measurable disease and are evaluable for toxicity and response. The median age of the patient population was 57 years (range, 16–68). Their median performance status (Karnof-

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sky scale) was 70% (range, 90%–50%). Thirteen males and three females were treated. Twelve patients had undergone a prior nephrectomy. Eleven patients were previously untreated except for primary surgical intervention at the time of initial presentation. Of the other five patients, two had received prior hormone therapy with androgens, two had received prior chemotherapy, and four had received radiotherapy (to a local lesion prior to chemotherapy). All previously treated patients had failed to respond to the prior treatment modality. The majority of patients had pulmonary (12 patients) or hepatic (seven) metastatic involvement. Other disease sites included: bone (four patients), adenopathy (two), brain (one), and skin (one). Thirteen patients had involvement of more than one metastatic site. Six patients had bone marrow biopsies performed while in the study and all biopsies were negative for tumor.

No patient showed regression of measurable disease after treatment with AMSA. Eleven patients had rapidly progressive disease despite therapy, four had initial stabilization (8–12 weeks) then progression, and one had stabilization of disease for 30+ weeks.

Toxic effects were mild and easily tolerated in all patients. Nonhematologic toxic effects consisted of nausea (seven patients, 43%), vomiting (five patients, 31%), phlebitis (one patient, 6%), and infection related to AMSA-induced granulocytopenia (two patients, 12%). There were no cardiac, neurologic, hepatic, or renal toxic effects observed.

The major toxic side effect of AMSA therapy is leukopenia. The median nadir wbc count for all courses was 3200/μl (range, 700–6400/μl) with a median day to nadir of 11 (range, 6–17). Seventy-five percent of patients had a median wbc count of $< 4000/\mu l$ with a median nadir for this group of 2200/μl. Four patients (25%) had wbc count nadirs of $< 1000/\mu l$. Recovery ($\geq 3000/\mu l$) in those applicable courses occurred on a median of 20 days (range, 14–28) from the AMSA treatment. Patients were re-treated on Day 21 if the wbc count was $\geq 3000/\mu l$. Three patients received AMSA escalations to 150 mg/m². The median wbc count nadir for the four evaluable courses at this level was 3500/μl with median days to nadir and recovery of 9 and 22, respectively. Other nonhematologic toxicity was not accentuated in the patients who received escalations compared with all of the patients in the study. Only one course at a dose of 120 mg/m² resulted in a platelet count of $< 100{,}000/\mu l$. No platelet transfusions were required and no hemorrhagic phenomena were observed in this study. The maximum number of courses of AMSA administered to a patient in this study was ten: three patients received one course; seven had two courses; four had three courses; and two had more than three courses. There was no evidence of any cumulative hematologic or nonhematologic toxicity. Although patients were not monitored for cardiac arrhythmias, no patient was observed to have had any cardiac symptomatology on any AMSA treatment day.

DISCUSSION

The present study failed to show any activity of AMSA when used as therapy for metastatic renal cell carcinoma. Although stabilization was achieved in some patients, the heterogenous behavior of this tumor would suggest that no significant antitumor activity was identified.

AMSA was well-tolerated. Nausea and vomiting was negligible and the myelotoxicity observed was easily handled by this largely untreated patient population. The myelosuppression of AMSA is moderate-to-severe leukopenia which is usually abrupt but of short duration (5–7 days). The hematologic data from this study confirm our phase I AMSA data (9) that a dose of 120 mg/m² is a safe level to conduct phase II studies in patients without extensive prior myelosuppressive therapy. There is a significant cohort of patients (at least 19% in this study) who can tolerate escalations to 150 mg/m² × 1 and perhaps higher.

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Combination Chemotherapy With Cyclophosphamide, Doxorubicin, and Bleomycin for Metastatic Transitional Cell Carcinoma of the Urinary Tract[1]

Brief Reports and Preliminary Communications

John A. Levi,* Rodney S. Aroney, and David N. Dalley[2]

SUMMARY

Twenty-three patients with metastatic transitional cell carcinoma of the urinary tract received at least two courses of the combination of cyclophosphamide, doxorubicin, and bleomycin. Eight patients (35%) achieved partial remission and an additional six (26%) had stable disease. Duration of survival was significantly longer for the partial responders (median, 30 weeks) than the remaining patients (median, 18 weeks). Principal morbidity (mucositis, fever, and pulmonary toxicity) was due to bleomycin. These results are not superior to single-agent chemotherapy and reinforce the need for further careful evaluation of new combination programs containing *cis*-diamminedichloroplatinum(II). The failure of bleomycin to improve responses and its considerable toxicity suggest that its continued systemic use in this disease is not warranted.

[Cancer Treat Rep 64:1011–1013, 1980]

Relatively few single agents have been adequately evaluated in the treatment of metastatic transitional cell carcinoma (1). Cyclophosphamide and doxorubicin (Adriamycin) have both demonstrated activity as single agents while in combination they were synergistic in a carcinogen-induced bladder cancer model in mice (2). Clinical studies with this two-drug combination, however, have produced conflicting results (3,4). Bleomycin has received minimal attention as a single agent given systemically but has definite efficacy when utilized intravesically in transitional cell carcinoma (5). Since bleomycin is well-suited for use in full doses in combination chemotherapy programs, the present study has further evaluated the combination of cyclophosphamide and doxorubicin and the potential efficacy of systemic bleomycin as part of the therapeutic regimen.

MATERIALS AND METHODS

Twenty-three patients with biopsy-proven metastatic transitional cell carcinoma of the urinary tract were evaluable in this study (table 1). The median age of patients was 61 years (range, 53–77 years). All patients had at least one measurable or evaluable site of disease to serve as an objective indication of response to therapy. Evaluable lesions including pelvic extension and iliac and paraaortic nodes were assessed by serial pelvic ultrasonography or computerized transaxial tomography. Bone and lung lesions were assessed by serial skeletal and chest radiography while the remaining patients had clinically palpable disease that could be measured in two diameters.

All patients received combination chemotherapy with cyclophosphamide (500 mg/m² iv, Day 1), doxorubicin (50 mg/m² iv, Day 1), and bleomycin (30 mg im, Day 1). The cyclophosphamide and doxorubicin were repeated at 21-day intervals while bleomycin was given weekly for a total of 12 weeks (maximum dose, 360 mg). Doses of the first two agents were modified, if necessary, according to the degree of myelosuppression observed with previous courses and, in the case of doxorubicin, if the serum bilirubin was elevated. Bleomycin doses were modified if skin and mucosal toxicity developed to any degree and as the toxicity abated doses were progressively escalated back to full levels.

Doxorubicin was deleted from the regimen when the cumulative dose reached 500 mg/m². Patients showing definite evidence of disease progression

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TABLE 1.—Patient characteristics and response to therapy

Characteristic	No. of patients treated	No. of partial remissions	(%)
Evaluable patients	23	8	(35)
Males/females	14/9	4/4	(29/44)
Site of primary tumor			
Bladder	16	7	(44)
Renal pelvis	6	1	(17)
Ureter	1	0	
Predominant metastatic site			
Pelvic extension	6	2	(33)
Regional and peripheral nodes	7	3	(43)
Lung	3	2	(67)
Liver	3	1	(33)
Bone	4	0	
Prior therapy			
None	7	2	(29)
Primary surgical resection	7	2	(29)
Radiotherapy ± surgery	9	4	(44)
Performance status*			
0–2	11	5	(45)
3–4	12	3	(25)

*Eastern Cooperative Oncology Group criteria.

after two courses of therapy were removed from the study while the remainder continued to receive regular courses of therapy until there was clear-cut evidence of progression or relapse.

Partial remission was defined as a > 50% decrease in the size of the principal measurable or evaluable parameters of disease with no new lesions appearing. Stable disease was defined as a < 50% regression of measurable or evaluable parameters of disease with no new lesions appearing. Progressive disease was defined as a > 25% increase in size of any pre-existing measurable or evaluable lesions, the appearance of new lesions, or the significant deterioration of weight and performance status.

RESULTS

Eight patients (35%) achieved a partial remission while an additional six (26%) had stable disease. Time to maximum response was generally short, with a median of 6 weeks (range, 3–12 weeks). Patients with a poor initial performance status tended to fare badly, with 67% having definite progression of disease despite chemotherapy. Predominant bone involvement was the only other adverse pretreatment prognostic factor; it was present in four patients, and none of them responded to therapy (table 1). Patients who achieved a partial remission had a significantly longer survival time ($P < 0.05$) (median, 30 weeks; range, 19–75+ weeks), and two patients are still alive at 58 and 75 weeks (median, 18 weeks; range, 2–54 weeks).

The most disabling toxic effects encountered were associated with bleomycin administration. Skin and mucosal toxic effects occurred in 13 patients and were severe in eight while fever was noted in six patients and was severe in two. Lung toxic effects occurred in three patients but abated in all after cessation of bleomycin. Myelosuppression was generally not severe, with only 3% of the courses producing a wbc count nadir of < 1000/μl and 16% producing a wbc count nadir of < 2000/μl. However, four episodes of severe infection occurred, with two resulting in death. Platelet counts of < 100,000/μl occurred in only 1% of the courses. Alopecia and nausea and vomiting were almost universal, and the latter was usually well-controlled with antiemetics.

DISCUSSION

The results of the present study are disappointing in that the 35% partial remission rate achieved, with an associated improvement in survival, is not clearly superior to results observed with the most active single agents, particularly *cis*-diamminedichloroplatinum(II) (DDP) (1,6). Indeed, other combination chemotherapy studies before the introduc-

tion of DDP also generally failed to demonstrate any definite advantage over single-agent chemotherapy (7,8).

The principal morbidity encountered was associated with bleomycin administration, and since this agent did not apparently contribute to any improvements in responses, its continued use in advanced transitional cell carcinoma is probably not warranted. Poor performance status and metastatic involvement of bone were unfavorable prognostic factors in this series and similar findings have also been reported by Yagoda et al (6,9). It will therefore be of importance in the evaluation of future controlled studies that stratification for these factors be specifically considered.

The preliminary reports of combination chemotherapy programs using DDP in advanced transitional cell carcinoma look potentially encouraging with quite high response rates (7,8). However, it is noteworthy that the three-drug combinations are not obviously superior to the two-drug combinations. In view of the previous failures to demonstrate an advantage for combination chemotherapy, further investigation of DDP combinations should probably initially concentrate on a properly stratified randomized study of DDP alone compared with a two-drug combination that includes DDP.

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LETTER

L-Phenylalanine Mustard, Doxorubicin, and Vincristine in Advanced Breast Cancer: A Pilot Study[1]

The combination of doxorubicin (Adriamycin) and vincristine (AV) has been reported to produce a response in 22%–28% of women with breast carcinoma previously treated with a combination of cyclophosphamide, methotrexate, and 5-FU (CMF) (1,2). Those reports also suggested that AV was non-cross-resistant with CMF. The alkylator, L-phenylalanine mustard, has been reported to be an active agent against breast carcinoma with responses in 18%–23% of patients (3,4). In addition, it has been suggested that L-phenylalanine mustard and cyclophosphamide may have different mechanisms of action and be at least partially non-cross-resistant (5). For these reasons we combined L-phenylalanine mustard (P) with AV to gain a preliminary impression of the activity of PAV. The results of this experience are reported.

MATERIALS AND METHODS

Seventeen patients with metastatic breast carcinoma receiving PAV were evaluated. Drug doses were: L-phenylalanine mustard, 3 mg/m² orally on Days 1–5; doxorubicin, 20 mg/m² (eight patients) or 30 mg/m² (nine patients) iv on Day 1; and vincristine, 1.2 mg/m² iv on Days 1 and 8 (maximum dose: 2 mg). Each treatment cycle was 28 days. All patients had progressive measurable and/or evaluable disease. Twelve patients were < 50 years old and 14 were postmenopausal. Disease dominance was visceral in eight patients, osseous in five, and soft tissue in four; 11 patients had one or two organ sites of involvement. The Karnofsky performance rating was 90–100 in two patients and ≤ 70 in three. Fifteen of the patients had received prior chemotherapy consisting only of CMF (11 responses, one failure, three not evaluable), and 13 had received prior hormonal therapy, including the two who had not received prior chemotherapy.

Clinical evaluations were performed at 1–2-month intervals using the Breast Cancer Task Force response definitions (6). Briefly, a complete response (CR) required clinical elimination of all measurable and evaluable disease for at least 4 weeks, a partial response (PR) required a ≥ 50% regression in at least 50% of the involved organ sites for at least 4 weeks, a no change (NC) required failure to achieve a PR or CR status at any time but without progression during the first 56 days, and progressive disease (PD) required a ≥ 25% increase in disease or the development of a new lesion.

RESULTS

Therapeutic effect.—There was one CR, two PR, nine NC, and five PD among the 17 patients, for a response rate of 18% (95% confidence interval, 4%–43%). The CR was in a patient receiving 20 mg/m² of doxorubicin, who was > 50 years old and postmenopausal with one soft tissue site of disease and no prior exposure to chemotherapy. The patients with PR were ≤ 50 years old and postmenopausal secondary to oophorectomies; one had osseous and soft tissue disease (three sites) and one had a single soft tissue site of involvement. One patient received the lower doxorubicin dose and one the higher dose. Both patients with a PR had received prior CMF therapy, one with a response and one as a postoperative adjuvant. The median time to treatment failure for all patients was 89 days, for those with PD 25 days, for those with NC 155 days, and for the three responders failure occurred at 84, 114+, and 326 days.

Side effects.—Side effects were equivalent across both levels of doxorubicin. Leukopenia of < 2000 cells/mm³ occurred in six of 17 patients, < 3000 cells/mm³ in 12 of 17, and < 4000/mm³ in 16 of 17. Platelet counts of < 100,000/mm³ occurred in five of 17 patients. There were no serious infections. Vincristine neurotoxicity was recorded in 11 cases with two cases requiring cessation of the drug due to weakness. One episode of congestive heart failure after 150 mg/m² of doxorubicin occurred in a 56-year-old patient.

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DISCUSSION

The present trial suggests that combining L-phenylalanine mustard with doxorubicin and vincristine (PAV) provides little, if any, therapeutic enhancement. The response rate to AV in patients previously treated with CMF would be expected to be 22%–28% (1,2) whereas that to PAV in the present trial was 18%. It is of interest that Rossi et al (7) reported a 23% response rate (11 of 47 patients) in a pilot trial comparing two PF (L-phenylalanine mustard and 5-FU) regimens with PF plus prednisone in previously untreated patients. Whether the results of these trials are related to dose reductions attendant with L-phenylalanine mustard-containing combinations or to a decreased effectiveness of L-phenylalanine mustard compared to other alkylating agents is not clear. Despite these uncertainties and the absence of a randomized trial, the present evidence suggests that combinations containing L-phenylalanine mustard are less active than cyclophosphamide-containing programs in advanced disease.

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